

**DYNAMIC ASPECTS OF
BIOCHEMISTRY**

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BY

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CAMBRIDGE
AT THE UNIVERSITY PRESS
1948

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
(Cambridge, and Bentley House, London)
Agents for Canada and India: Macmillan
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First Edition 1947
Reprinted 1948

To

HOPPY

(Sir Frederick Gowland Hopkins, O.M., F.R.S.)

WITH RESPECT, ADMIRATION
AND AFFECTION

'The difference between a piece of stone and an atom is that an atom is highly organised, whereas the stone is not. The atom is a pattern, and the molecule is a pattern, and the crystal is a pattern; but the stone, although it is made up of these patterns, is just a mere confusion. It's only when life appears that you begin to get organisation on a larger scale. Life takes the atoms and molecules and crystals; but, instead of making a mess of them like the stone, it combines them into new and more elaborate patterns of its own.'

ALDOUS HUXLEY, *Time must have a Stop.*

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PREFACE

IN spite of war-time difficulties and restrictions, Biochemistry has continued to expand more and more rapidly each year, in stature as well as in scope. It has been impossible for many years past for any one worker to read more than a small fraction of the new output, even when foreign journals were available. Without the invaluable aid of the *Annual Reviews of Biochemistry*, to whose authors and editors I and every other biochemist must pay high tribute, the preparation of this new book would have been impossible. Even with their help, some sections of the book will probably be out of date by the time it appears in print, and may well be out of date already in certain respects. But the last few decades have seen the establishment of a considerable body of information which, though it may change considerably in detail, will perhaps not change significantly in substance during the next few years. I venture to hope that a new edition may be called for before the present contents have become wholly archaic, so that there will be opportunity to correct the many faults which have doubtless escaped notice and to bring the whole volume up to date.

The subject-matter of biological chemistry, like that of biology itself, can be roughly divided into two parts; the static, or morphological, and the dynamic, or physiological. Knowledge of the latter demands as an essential pre-requisite a knowledge of the former, and it is therefore a matter for rejoicing that many organic chemists of the present day are devoting their attention to the chemical constitution and configuration of the organic *Bausteine* that form the material basis of living cells. In some fields, notably in that of protein chemistry, the interdependence, and collaboration between the organic and the biochemist are so intimate that it is impossible to say which is the organic chemist and which the biochemist. If any differentiation were necessary it would best be made, probably, in terms of their respective attitudes towards the *Bausteine*. For the organic

chemist the main focus of attention is the structure and configuration of these materials while, for the biochemist, the main problems are those of the behaviour and function of these substances in organized, biological systems.

The more static aspects of these *Bausteine* are already fairly well covered by monographs and review articles, some of which I have indicated in the bibliography; the essentially dynamic aspects, on the other hand, have hitherto been but inadequately described and, in view of their wide importance and interest, I believe that a real demand exists at the present time for a book of this kind.

Elementary Biochemistry is taught in this University in two courses. The first and older of these, Chemical Physiology, forms part of the course in mammalian Physiology, and caters primarily for the needs of medical and veterinary students. For these there already exists a wealth of text-books, which the present volume neither hopes nor desires to supplant. In the second course, much more recently introduced, Biochemistry is taught as an independent scientific discipline, and without that emphasis on clinical problems with which it has usually been associated in the past, and which properly finds a place in Chemical Physiology. For students taking this second course there exists no suitable text-book, and it is primarily with their needs in mind that the present book has been written. I hope, however, that it will also help to open up new horizons to those whose interest in Biochemistry is primarily that of the organic chemist or the clinician in training. Perhaps it will serve too as an introduction to others who, wishing to take an advanced degree course here or elsewhere, find it difficult at the present time to discover suitable elementary reading. With the needs of such students as these in mind I have included a short bibliography of review articles and books, mostly of recent date and written by experts in their respective fields.

In Biochemistry, as in any young but rapidly expanding branch of science, there are fields in which facts are scanty, evidence contradictory and speculation rife. I have tried to avoid such topics, but where this has not been possible I have

attempted to give a critical account of the facts but, at the same time, to speculate a little. I cannot wholly subscribe to the doctrine that speculation is out of place in an elementary textbook, for there are many gaps in the subject, and unless these can in some way be bridged it is difficult or impossible to give a coherent account. My experience as a teacher has been that coherence is essential in an elementary exposition. Speculation plays and has always played an important part in the advancement of scientific knowledge, for no research worker gropes blindly after he knows not what; he invariably begins with certain reasonable possibilities in mind. In short, he speculates. To speculate unreasonably is worse than not to speculate at all, but providing certain tests of reasonableness and compatibility are applied beforehand, speculation is a valuable tool, and one which finds a place in every scientific workshop. The danger is that speculation is not always recognized as what it is, and I have, therefore, tried to distinguish clearly between fact and fantasy, hoping in this way to steer a middle course between unbridled imagination on the one hand, and an equally undesirable hypertrophy of the critical faculty on the other.

A word of explanation is perhaps necessary for the use in these pages of somewhat novel and certainly unorthodox methods of writing the equations of certain chemical reactions and groups or sequences of reactions. I have adopted them only after a long period of trial. They give a distinctively pictorial representation of chemical events, and many students find such a picture more easily comprehended and remembered than the more formal representations usually adopted. I trust that the reader will exercise the little patience necessary to become familiar with these 'whirligigs', for they have great advantages in cases where a long chain of successive chemical events has to be described briefly and as a whole.

The writing of this book has been largely a spare-time occupation, and there has been little enough spare time during the war years. Progress has often been slow, therefore, and the task has nearly been abandoned more than once. I owe the fact of its

eventual completion to the kind encouragement given to me by my friends and colleagues. Particular thanks are due to Dr D. J. Bell and Dr E. Watchorn, who have read the whole of the manuscript, and to Prof. A. C. Chibnall, who read the proofs. I am glad also to acknowledge the help I have had from Miss V. Moyle. These, and others who have read particular sections and chapters, have all given precious advice and valuable criticisms. My task has been simplified in many ways by Prof. J. B. S. Haldane's *Enzymes* and by Dr D. E. Green's *Mechanisms of Biological Oxidations*, and particular thanks are due to Dr Malcolm Dixon, who has given me much from his great personal store of information.

I should also like to record my thanks to Dr J. C. Boursnell, who heroically undertook the preparation of the index, to Mr H. Mowl, who prepared the drawings for Fig. 13, and to members of the Cambridge Part I Biochemistry Class of 1945-6, who have allowed me to make use of some of their experimental data in the preparation of Figs. 1, 3 and 7.

To my wife, who prepared the work for publication, and to all departments of the Cambridge University Press I wish to express my humble and hearty thanks for their patience, consideration and expert workmanship.

E.B.

CAMBRIDGE

January 1948

ACKNOWLEDGEMENTS

The author's thanks are due to the following for permission to reproduce figures: the Cambridge University Press for Figs. 2, 3C, and 23; Dr H. Fraenkel-Conrat and the *Journal of Biological Chemistry* for Fig. 3B; Drs F. Schlenk and F. Lipmann and the University of Wisconsin Press for Figs. 20, 21 and 30; Messrs Longmans Green & Co., Ltd. for Figs. 4, 5, 9, and 12; and Messrs W. Heffer & Co. Ltd. for Fig. 11.

PART I

ENZYMES

CHAPTER I

THE GENERAL BEHAVIOUR AND PROPERTIES OF ENZYMES

INTRODUCTION

WHEREVER we turn in the world of living things we find chemical changes taking place. Green plants, together with certain bacteria, are capable of fixing solar energy and synthesizing complex organic substances of high-energy content from very simple starting materials, namely, water, carbon dioxide and small amounts of inorganic substances such as nitrates and phosphates. Other living organisms possess the ability to decompose these complex materials and to exploit for their own purposes the energy that is locked up within them, and it is in this way that animals, for instance, obtain the energy they expend in the discharge of their bodily functions, reproduction, growth, locomotion and so on. Now it is a significant fact that nearly all the chemical changes that go on in living tissues are changes which, in themselves, proceed too slowly to be measurable or even, in many cases, detectable. How, then, does it happen that living animals can obtain energy and expend it as fast as they do? The answer is that living organisms possess numerous catalysts which speed up chemical reactions to the rates achieved in biological systems. Whether we consider digestion, metabolism, locomotion, fermentation or putrefaction, chemical changes are going on, and these chemical changes are catalysed. It is the purpose of this book to give some account of these changes and of the various mechanisms at present known to participate in their catalysis.

A catalyst, in the classical definition of Ostwald, is 'an agent which affects the velocity of a chemical reaction without appearing in the final products of the reaction'. Examples of catalysts are familiar to every student of chemistry, and perhaps the most striking is that commonest of all chemical reagents, water. As is well known, hydrogen and chlorine react together with explosive violence if exposed to sunlight, and yet, as Baker showed, perfectly dry hydrogen and perfectly dry chlorine fail to react together at all. Baker found that numerous familiar reactions do not proceed except in the presence of traces of water, and that water is, in fact, a very important catalyst. Finely-divided metals, such as platinum, nickel and palladium, also are capable of catalysing a wide range of reactions, and Wieland, for instance, found that on the addition of colloidal palladium to aqueous solutions of various simple organic compounds, a catalytic oxidation (dehydrogenation) of the compounds ensues. Many more examples could be cited. Thus the hydrolysis of esters is a process that goes on very slowly in neutral solutions but is greatly accelerated by traces of strong acids or alkalis. Again, chemical reactions as a whole proceed more rapidly at higher than at lower temperatures. But living organisms do not have at their disposal the strong acids and alkalis, the high temperatures and the other artifices which are available to a chemist working in a laboratory, yet the synthetic ability of living cells and tissues far surpasses that of the chemist.

Before we can deal satisfactorily with the problem of catalysis it is necessary to know something about the conditions which determine whether or not any given chemical reaction can take place. To analyse these conditions completely would require somewhat lengthy thermodynamic arguments, but for present purposes use may be made of a simple mechanical analogy. Let us consider a perfectly smooth body standing on a perfectly smooth plane. This body has a certain amount of gravitational potential energy, but this energy is not available for the performance of work of any kind unless the plane is tilted. Suppose now that the plane is slightly inclined. The body begins to slide downwards

because some of its potential energy has become available to push it down the plane. When the body slips, work can be done (e.g. if the body is attached by means of a string to some suitable motor), and the amount of work done will be thermodynamically equivalent to the amount of gravitational potential energy lost by the body. How much work can be done by this system depends upon the system itself, for while it is theoretically possible for the body to go on sliding down an inclined plane of indefinite length until the whole of its potential energy has been converted into work, this is not a case of much practical interest. Generally speaking the properties of natural systems are such that only a part of their total potential energy is available for the performance of work. A larger or smaller part of the total energy will be unavailable except in theoretical cases. Hence we must distinguish between the 'free' or available energy and the total energy of the system.

Now we know as a matter of practical experience that a body will always slide *down* an inclined plane if left to its own devices, provided that the frictional forces opposing the tendency to slip are not too great. If these forces are reduced to zero the body will certainly slip, and work can be done by the system. The body will never move *up* the plane so long as the system is left to itself; an upward movement can only be accomplished by supplying energy to the system from an external source. It follows, therefore, that in any self-operating machine, the free energy will always tend to decrease and, providing that frictional forces do not oppose it, the whole of the free energy lost to the system will be converted into an equivalent amount of work.

We can express these ideas more precisely with reference to heat engines. Let us consider a heat engine supplied with an amount of energy equal to Q . This energy is supplied at a high temperature T_1 (measured on the absolute scale), and conducted to a lower temperature T_2 . It can be shown on theoretical grounds that the amount of work done by such an engine cannot exceed an amount W , where

$$W = Q \frac{T_1 - T_2}{T_1}.$$

This equation can be transformed to give

$$W = Q - \left(\frac{Q}{T_1}\right) T_2.$$

Thus the maximum available work will always be less than the total possible work by an amount given by $(Q/T_1) T_2$. It follows that an engine of this kind can only convert its total energy into useful work if $(Q/T_1) T_2 = 0$, i.e. when $T_2 = 0$, and the 'exhaust' or condenser of the engine is maintained at the absolute zero of temperature. At all other temperatures a part of the total energy will be unavailable, and the magnitude of the unavailable energy at any temperature T is determined by the product of that temperature and the factor (Q/T_1) . The latter is known as the entropy of the system and is usually represented by S . Entropy measures the extent to which the total energy of the system is unavailable for the performance of useful work.

Similar considerations apply to systems other than heat engines, and for any self-operating system working at a temperature T we can write the following general equation:

$$F = H - T.S,$$

where F represents the free energy of the system (sometimes represented by G), i.e. the amount of energy available for the performance of useful work, H is the total heat energy of the system and S is its entropy. It is not possible to determine the absolute values of these variables apart, of course, from the temperature. We can, however, observe the changes that they undergo when the system passes from its original state into a new condition represented by

$$F' = H' - T.S'.$$

Subtracting from the former equation we get

$$(F - F') = (H - H') - T (S - S'),$$

or, in the usual terminology,

$$\Delta F = \Delta H - T.\Delta S.$$

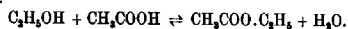
Now chemical changes as a whole are accompanied by thermal changes, and are most commonly exothermic. But the heat evolved during an exothermic process does not represent the change of free energy, for the change of entropy has also to be reckoned with. In an ordinary chemical reaction, therefore, all we can measure directly is ΔH , the change in total heat energy, and ΔF can only be arrived at indirectly. Sometimes the change of entropy is small, so that ΔF is approximately equal to ΔH , but it is equally possible to have a reaction in which the change of entropy is very large indeed, so that the reaction is actually endothermic, but nevertheless results in a loss of free energy.

It is most important, therefore, to remember that the total heat change of an exothermic or endothermic reaction gives no indication as to whether or not that reaction takes place under its own power or is assisted by other processes going on in the system; the only reliable guide is a knowledge of ΔF . *If ΔF is zero the system is in chemical equilibrium: if it is positive (i.e. free energy goes into the system) the reaction cannot take place except with external aid: and if it is negative (i.e. free energy comes out of the system) the reaction can proceed of its own accord.*

A chemical reaction is thermodynamically possible, then, if it is attended by a loss of free energy. Whether or not it actually takes place, however, depends upon other factors. A body will not slide down a rough plane if the frictional forces are greater than the forces exerted by its free gravitational potential energy. Similarly, while a chemical reaction *can* take place if the reactants have sufficient free energy, it will not actually *do* so if the 'frictional forces' tending to oppose it are too large. In other words, *a chemical reaction requires for its accomplishment that the molecules shall be in a reactive state.* Thus we may keep a neutral, aqueous solution of sucrose almost indefinitely without appreciable hydrolysis, for although hydrolysis is thermodynamically possible, it does not actually take place because the molecules are not sufficiently reactive. If we add a small amount of saccharase or, alternatively, a little dilute mineral acid, the sugar is hydrolysed. By 'activating' the molecules these catalysts overcome the 'frictional forces' opposing hydrolysis.

It follows from all this that a catalyst cannot initiate a reaction that is not already possible on energetic grounds: all that it can do is to influence the velocity at which a thermodynamically possible reaction actually takes place.

We must know something about simple catalysts and their mode of action before turning to the more complex catalysts and catalytic systems that we find in living tissues. There are many resemblances between catalysis as effected by more or less complex chemical reagents on the one hand and by biological systems on the other, but differences also exist. In the first place, a catalyst, of whatever kind, only affects the *rate* of the reaction which it catalyses. This fact is particularly well illustrated in the case of a reaction such as the hydrolysis of an ester, which is reversible. If we take ethyl acetate, for example, and heat it with water, the ester is slowly hydrolysed, but the reaction stops before the hydrolysis is complete. On the other hand, if we start with equivalent proportions of ethyl alcohol and acetic acid and heat these together, we find that they react to form ethyl acetate, but once again the reaction stops before reaching completion. Indeed, from whichever side we start, the composition of the final reaction-mixture is always the same, the system attaining a state which can be represented by the following equilibrium:



If we employ dilute mineral acid as a catalyst we again obtain a reaction-mixture of the same composition, while if a biological catalyst such as liver esterase is used, the final composition is the same once more. These facts point to several important conclusions: first, that only the reaction velocity, and not the extent to which the reaction proceeds, is affected by the catalyst, and secondly, that in the case of a reversible reaction (and on theoretical grounds it is usual to assume that all reactions are reversible) the catalyst influences the reaction velocity equally in both directions. The direction in which such a reaction will proceed is determined, of course, by mass-law considerations and by the availability of free energy. We must therefore suppose that a catalyst which accelerates the decomposition of a given

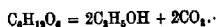
substance must also be capable of catalysing its synthesis. But it does not by any means follow that the necessary conditions can be experimentally realized.

A second important feature of the phenomenon of catalysis is that the effect of the catalyst is normally out of all proportion to the amount used. A minute quantity of colloidal platinum is sufficient to catalyse the decomposition of an unlimited amount of hydrogen peroxide, provided nothing happens to interfere with its catalytic properties. In practice, however, it frequently happens that catalysts are inhibited ('poisoned') by the presence of extraneous material. Thus in the example just given, minute quantities of hydrocyanic acid, mercuric chloride or certain other substances suffice to destroy the catalytic properties of the platinum. This 'poisoning' is often a serious nuisance in commercial processes, but in many cases the catalytic activity can be recovered relatively easily. In biological systems, too, we find that a comparatively small concentration of the catalytic material is all that is necessary, and that the catalysts are easily inhibited in a variety of ways which we shall discuss in later sections.

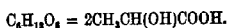
According to Ostwald's definition, the amount and chemical composition of a catalyst is the same at the end of its period of activity as it was at the beginning, though it is frequently found that its physical properties have been changed. Here is what at first sight appears to be a fundamental difference between catalysts such as colloidal metals and the catalytic agents we find in living tissues, but the difference is more apparent than real. Biological catalysts commonly lose much of their activity as the reactions which they catalyse proceed, but in such cases it usually appears that the catalyst has undergone inhibition by the products of its own activity, or else that its physical state has been modified in such a way that its catalytic properties have been destroyed.

Another apparent difference between the two types of catalysts is that whereas a catalyst such as platinum black does not initiate a reaction but only accelerates one which already proceeds, albeit very slowly, in its absence, biological systems do

in certain cases appear to initiate new processes. For example, living yeast cells catalyse an almost quantitative conversion of glucose into ethyl alcohol and carbon dioxide according to the well-known equation:



By contrast, certain bacteria, e.g. *Streptococcus faecalis*, catalyse the conversion of glucose into lactic acid:



Glucose itself does not show any propensity to decompose spontaneously into either alcohol or lactic acid. Nevertheless, there is no real theoretical difficulty here. As is well known, it is the rule rather than the exception in organic chemistry that side-reactions take place, indicating that organic substances tend to decompose or react in more ways than one. Let us suppose, therefore, that glucose can decompose into a series of different products, *A*, *B*, *C*, *D* and so on, each product arising by its own series of reactions. Under ordinary conditions the conversion of glucose into *A*, *B*, *C*, etc., proceeds only at imperceptible speed, but, under the influence of the catalysts of yeast, one of the possible modes of breakdown is selectively accelerated to such an extent that it is followed almost quantitatively. The catalysts of *S. faecalis*, by contrast, selectively accelerate another and a different mode.

This last case serves to illustrate what is perhaps the most striking feature of biological catalysis. Whereas a catalyst such as platinum black can catalyse any of a rather wide range of reactions, it is characteristic of biological systems that they catalyse only one kind of reaction and even, in many cases, one particular reaction and one only. But this is a difference only in their degree of specificity, or exclusiveness, and cannot be reckoned as evidence that biological catalysts differ essentially or fundamentally from catalysts of other kinds.

Although the effects of biological catalysts have long been familiar, and although they have been deliberately used by mankind since the dawn of history for the production of cheese, alcoholic beverages and the like, it is only in comparatively

recent years that we have acquired any knowledge or understanding of their mode of action. The celebrated Italian physiologist, Spallanzani, was perhaps the first to make a deliberate study of one of these catalysts, and this he did by feeding hawks with pieces of meat enclosed in wire cages, which were later regurgitated. In this way he demonstrated that the gastric juice of hawks contains something which brings about the liquefaction of meat. But as yet the nature of the responsible agent, which we now know under the name of pepsin, could not even be guessed.

It was Louis Pasteur who laid the foundation of our present knowledge. In the course of his famous researches on fermentation he demonstrated that solutions of organic materials such as glucose are perfectly stable if carefully sterilized and stored in sealed vessels. If, however, air was allowed to gain access to the solutions, fermentation set in, and this, Pasteur showed, was due to contamination with living yeast cells which came in with the air. So long as these micro-organisms were carefully excluded, no fermentation took place. Similarly, Pasteur showed that the souring of wine, a troublesome phenomenon which he was commissioned by the then government of France to investigate, was attributable to the presence of certain other micro-organisms. These and other observations of a like kind led Pasteur to conclude that processes such as alcoholic fermentation, the souring of wine and milk are due to, and inseparable from, the vital activities of certain particular micro-organisms, which he accordingly named 'ferments'.

Pasteur's views received a severe blow when it was discovered by the brothers Buchner that if yeast is macerated with sand and submitted to high pressures, a juice can be expressed from it which contains no living cells whatever, but is nevertheless capable of fermenting sugar with the production of alcohol and carbon dioxide. The Buchners, in fact, succeeded in demonstrating what Pasteur regarded as an impossibility, the fermentation of sugar in the complete absence of living cells. Yeast juice clearly contains the catalyst or catalysts by means of which living yeast accomplishes the alcoholic fermentation of sugar,

and to describe this catalytic agent the term 'enzyme' was coined, from the Greek ἐν ζύμῃ, literally 'in yeast'. When other similar catalysts were later discovered and studied, the term enzyme was taken over as a collective title and the yeast-juice enzyme received the distinguishing name of zymase.

The discovery of zymase was a fundamental advance. It had hitherto been possible to study fermentation and kindred processes only in the presence of living cells, but living cells multiply, die off, use up some chemical substances and excrete others so that, superposed on fermentation proper, there are many other chemical processes. With the newly discovered yeast juice, however, the chemistry of fermentation could be studied in isolation, quite apart from all the other chemical operations carried out by the intact organism. As we now know, 'zymase' is not a single enzyme or catalyst, but rather a complex system of catalysts, and similar juices can be prepared from many kinds of cells. The Buchners made their fundamental discovery as recently as 1897, and progress thereafter was rapid. The first enzyme to be obtained in the pure, crystalline state was obtained only some 30 years later, in 1926, and since that time numerous others have been isolated and purified.

Certain important discoveries were made comparatively early in the rather meteoric history of enzyme chemistry. Thus it was found that zymase loses its activity completely if boiled, and that if it is dialysed its activity is similarly lost. After dialysis, though not after boiling, activity could be restored by adding the dialysate, i.e. the small-molecular materials removed by the process of dialysis, or by the addition of a little boiled yeast juice. These observations show that, in addition to the thermolabile, non-dialysable enzymes, yeast juice also contains thermostable, dialysable factors in the absence of which fermentation cannot go forward. Thus there arose the conception of enzymes as thermolabile substances of high molecular weight, and of a second group of catalytic materials, called co-enzymes, which consist of small, thermostable molecules. Both are necessary if fermentation is to take place. Just as we know that zymase is in reality a complex mixture of enzymes, so, too, the

dialysable complement is known to contain more than one co-enzyme, and we shall have a great deal to say about this particular case in a later chapter.

Even this brief review has revealed a number of the most important properties which characterize enzymes. *They are colloidal materials of high molecular weight, are thermolabile and highly specific, and can usually be extracted from the cells in which they are produced.*

NOMENCLATURE AND CLASSIFICATION OF ENZYMES

Enzymes may be classified in any of several ways. All enzymes, so far as we know, are produced inside living cells, and the majority of them do their work inside the cells which produce them, though they can usually be extracted and their activity studied independently. In simple animal organisms such as *Amoeba* the processes of digestion are preceded by the phagocytic ingestion of food particles, which then undergo intracellular digestion, but in more highly organized forms of animal life it is commonly found that digestive enzymes are secreted into the digestive cavity, so that digestion is extracellular, at any rate in part. Thus we can distinguish between intracellular and extracellular enzymes. This mode of classification is often useful.

More usually, enzymes are named and classified in terms of the reaction or reactions which they catalyse, though no practicable system of rigid nomenclature has yet been devised. We can distinguish, for example, a large and important group of enzymes which catalyse the hydrolysis of their substrates, i.e. the substances upon which their catalytic influence is exerted, and these enzymes are accordingly termed *hydrolases*. This group includes all the extracellular enzymes concerned with digestion, and many intracellular enzymes besides. Individual enzymes are usually named by adding *-ase* to the names of their respective substrates; for example, enzymes which catalyse the hydrolysis of starch are collectively called amylases (*amylum* = starch, Latin), and different individual amylases are distinguished by reference to the sources from which they are obtained. Thus we find

salivary and pancreatic amylases among the digestive enzymes of the mammals. Similarly, enzymes which catalyse the hydrolysis of proteins are known as proteinases, and those which act upon fats as lipases. The group of hydrolases also includes many non-digestive enzymes, such, for instance, as urease, which catalyses the hydrolytic breakdown of urea into ammonia and carbon dioxide, and arginase, which catalyses the hydrolysis of the amino-acid arginine into ornithine and urea.

A second large and important group of enzymes comprises those which catalyse biological oxidations and reductions. Most biological oxidations, as we shall see, involve the removal of hydrogen from the substrate undergoing oxidation, and most of the oxidizing enzymes are accordingly known as *dehydrogenases*. Some of these differ from the rest in certain respects and are called *oxidases*, though this must not be supposed to imply that they catalyse the addition of oxygen to their substrates.

In addition to these two large groups we know of a number of other types and, in what follows, we shall consider enzymes under the following headings:

- (1) *Splitting enzymes* (e.g. hydrolases).
- (2) *Oxidizing and reducing enzymes* (e.g. dehydrogenases).
- (3) *Adding enzymes*.
- (4) *Transferring enzymes*.
- (5) *Isomerizing enzymes*.

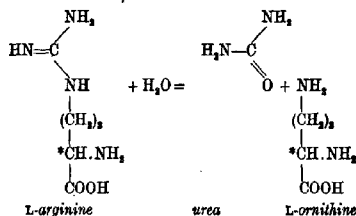
SPECIFICITY

One of the most striking properties of enzymes is their specificity. By this we mean that a given enzyme can catalyse only a comparatively small range of reactions, and even, in many cases, one reaction and one only. It is possible to distinguish fairly sharply between a number of different degrees and types of specificity, and to make this clear we may consider first of all what is known as optical, or better, as *stereochemical specificity*.

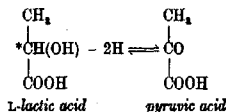
The majority of chemical substances formed and broken down in metabolic processes are optically active and, of the two possible stereo-isomeric forms in which such substances can exist,

only one is usually found on any large scale in natural materials and processes. Of the sugars, for example, we normally find only the D-isomers, though it is true that their enantiomorphs are very occasionally met. Thus L-galactose has been isolated from various plant materials and from the molluscan polysaccharide, galactogen. None the less, it remains a fact that there is an overwhelming preponderance of D-sugars in nature. Similarly, of the α -amino-acids only the L-members occur extensively in nature: cases of the occurrence of D-amino-acids have been reported, but are relatively rare. Perhaps, therefore, it is not surprising to find, as we do, that most enzymes show a strong and usually a complete selectivity for one member of a pair of optical isomerides, and are therefore said to exhibit stereochemical specificity.

The phenomenon of stereochemical specificity is well illustrated in the case of the hydrolytic enzyme arginase. This enzyme acts upon L- but not upon D-arginine, producing L-ornithine and urea:



Similarly, the lactic dehydrogenase of muscle can catalyse the dehydrogenation of L-(+)- but not that of D-(-)-lactic acid to yield pyruvic acid:



The same enzyme can also work in the reverse manner, acting upon the optically inactive pyruvic acid (asymmetric carbon atoms are marked with asterisks) and catalysing its reduction

to yield L-lactic acid only. D-Lactic acid is never formed by this enzyme. In many micro-organisms, however, we find a lactic dehydrogenase which is specific for the D-form of lactic acid, and this is true, for example, of *Bacillus delbrückii*, an organism that is employed for the commercial production of lactic acid.

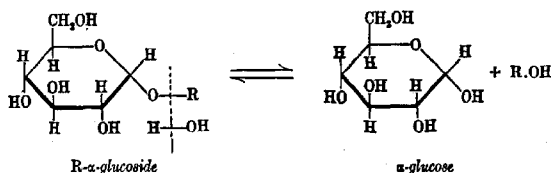
Over and above the stereochemical specificity which is to be observed in the majority of enzymes, other types of specificity can be recognized. These other types differ mainly in the degree of exclusiveness. If, for the sake of simplicity, we consider only hydrolytic enzymes for the moment, the reaction catalysed by any given enzyme can be represented thus:



The molecule of the substrate can be considered as consisting of three characteristic fragments, the two parts of the molecule itself, *A* and *B*, and the linkage which joins them. Three main types of specificity can be described with reference to these constitutional fragments. In the first type only the nature of the linkage is important, in the second the linkage and one-half of the molecule must be 'right', while in the third type all three fragments must be 'right'.

In the first type the precise nature of *A* and *B* is relatively unimportant, except that, if they are derived from optically active compounds they must have the appropriate stereochemical configuration, a condition which is already imposed by the stereochemical requirements of the enzyme. What is important, however, is that the linkage joining *A* to *B* shall be of the right kind, i.e. it must be an ester linkage in the case of a lipase or an esterase, a peptide link in the case of a peptidase, or a glycosidic link in the case of a glycosidase. If an enzyme is specific only towards the nature of the linkage bond it is said to exhibit *low specificity*. This type of specificity is less common than was once believed, for a number of enzymes of low specificity have proved to be mixtures of enzymes, each member of which is more specific than the original complex. It is found, however, among the lipases, which have so far defied all attempts to fractionate them.

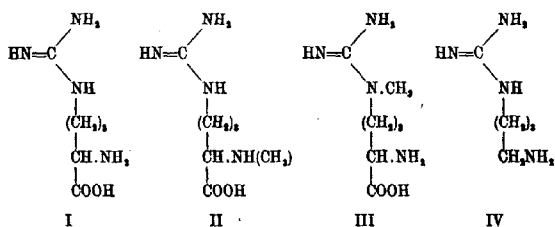
The second type of specificity is more exclusive, for here the enzyme can only act upon substances in which the right chemical linkage is present and in which one of the two parts, *A* and *B*, is also of the right kind. As an example we may consider the case of the digestive enzyme usually called 'maltase', since it catalyses the hydrolysis of maltose (glucose-4- α -glucoside). Maltase obtained from the intestinal juices of a mammal will catalyse other reactions however; its action upon maltose is only one example of its catalytic action upon α -glucosides in general, which may be expressed in the following manner:



The specificity requirements of this particular enzyme are as follows. An α -glycosidic link is required in the substrate, and compounds containing a β -glycosidic linkage are not attacked. Furthermore, the glycosidic radical must be derived from D-glucose, and replacement of the glucose unit by one derived from another sugar yields a product which is resistant to this particular enzyme. Thus the nature of the linkage *and* that of one-half of the molecule must be 'right' in every detail, though the nature of the 'R' group is a matter of relative indifference. An enzyme of this kind may be said to show *group specificity*, to indicate that it can act upon a group of closely related substrates, in this case a group of α -glucosides. Strictly speaking, therefore, this particular enzyme ought not to be called 'maltase', since its action is not uniquely confined to maltose; it is, in fact, an α -glucosidase. This kind of specificity is common among carbohydrases, for there also exist β -glucosidases, β -galactosidases and so on, each demanding its own particular kind of glycosidic linkage, together with a sugar radical of the appropriate type.

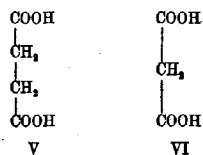
The third and commonest kind of specificity is the most exclusive of all. It is well illustrated by the maltase of

germinating barley (malt). Unlike the so-called 'gut maltase', malt maltase acts only upon maltose itself, and is without action where other α -glucosides are concerned, so that in this case the title of maltase is strictly applicable. Both parts of the substrate molecule must be 'right' in this case, together with the linkage bond, and the enzyme is therefore said to show *absolute specificity*. To take another example we may consider arginase again. This enzyme requires for its action that the substrate shall consist of unmodified L-arginine (I). Many substances derived from and closely related to L-arginine have been prepared and submitted to the action of this enzyme, but it fails always to act. Thus α -N-methyl arginine (II), δ -N-methyl arginine (III) and agmatine (IV) are all unaffected by arginase. Urease similarly requires that the structure of its substrate, urea, shall be intact and unsubstituted, and none of the considerable number of derived ureas that have been tested has been found to undergo hydrolysis under its influence.



Most of the examples so far mentioned have been chosen from the group of hydrolytic enzymes, but similar phenomena are to be seen in other groups. Thus succinic dehydrogenase acts only upon succinic acid (V) and is without action upon the closely related malonic acid (VI) by which, indeed, it is strongly inhibited. Succinic dehydrogenase is therefore absolutely specific, like malt maltase and arginase. Some oxidizing enzymes, however, are group-specific, and as an example we may take the case of the aldehyde oxidase of liver. Given suitable conditions, this enzyme can catalyse the dehydrogenation of many different

aldehydes, but its action does not extend to other groups of compounds such, for instance, as the alcohols.



In conclusion we may consider a very unusual case. Milk contains the so-called Schardinger enzyme, which catalyses the oxidation of a very large number of different aldehydes to yield the corresponding acids, and is therefore group-specific. But milk also contains a factor which catalyses the oxidation of hypoxanthine and xanthine to uric acid, and this factor has received the name of xanthine oxidase. Many purines other than hypoxanthine and xanthine have been submitted to its action and found not to be attacked, so that the specificity of xanthine oxidase is very nearly absolute. The curious fact is that the Schardinger enzyme and xanthine oxidase are demonstrably identical, so that in this case we have an enzyme which possesses two widely different ranges of specificity, one with respect to aldehydes, for which it is group-specific, and another with respect to purines, for which its specificity is virtually absolute. This instructive example makes it clear that the specificity of any given enzyme cannot necessarily be assigned to one or other of the types we have discussed. Low, group and absolute specificities are merely convenient standards of reference; many intermediate grades exist, just as, in the solar spectrum, we can distinguish between red, orange, yellow, green, blue and violet, although the colours themselves merge into one another and form a continuous whole.

Finally, and most important of all, there is the fact that specificity is in reality a measure of the structural specialization that an enzyme requires in its substrate, and this must probably argue a corresponding degree of structural specialization in the enzyme itself.

THE CHEMICAL NATURE OF ENZYMES

The fact that enzymes are not dialysable long ago suggested that they might be related to substances of high molecular weight such as the polysaccharides and proteins, and even before any enzyme had been obtained in the pure state there was a considerable mass of evidence that they are proteinaceous in nature.

In recent years many enzymes have been concentrated, purified, and finally isolated in pure, crystalline form, and in every case the product has proved to be a protein. Many enzymes, such as pepsin, trypsin and a number of other hydrolytic enzymes, consist wholly of protein, but in other cases, notably among oxidizing enzymes, there is attached to the protein part of the molecule a non-protein fragment of some kind, so that the enzyme is, in fact, a conjugated protein. While there is thus no doubt that every enzyme so far isolated is a protein of some kind, we cannot state categorically that all enzymes are proteins, if only because relatively few have so far been isolated. But even in other cases there is a good deal of indirect evidence to point to the proteinaceous nature of enzymes in general, and some of this we must consider here. Information regarding the chemical nature of enzymes has been obtained in many different ways, and most of it from considerations of the influence upon enzymic activity of environmental conditions such as temperature, pH and the presence of foreign materials of various kinds.

(i) *The Measurement of Enzymic Activity.* The activity of an enzyme is determined by measuring the amount of chemical change it catalyses under any given set of conditions. If we incubate an enzyme together with its substrate under suitable conditions of temperature, pH and so on, we can withdraw samples of the reaction mixture from time to time and follow the course of the reaction by analysing the samples. Thus, if we choose yeast saccharase as our enzyme and sucrose as the substrate, we can measure the amount of chemical change at any given moment in terms of the amount of reducing sugars (glucose and fructose) that has been formed from the original non-reducing sugar. It is usually necessary at the same time to carry

out a 'control' experiment in which the enzyme is replaced by a previously boiled sample of enzyme; in this way we can correct our experimental results for any changes that are due to spontaneous transformation of the substrate or to any other process that is not catalysed by the enzyme. Similar methods can be devised and used for the study of other enzymes, and the results of a typical experiment are shown in Fig. 1.

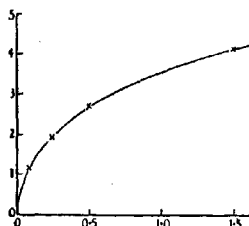


Fig. 1. A typical 'progress curve'; tryptic digestion of casein; data from a class experiment. Ordinate: increase in formol titre (ml. NaOH). Abscissa: time (hr.).

It will be observed that the reaction velocity soon begins to decrease and eventually the process stops altogether. Now while the reaction is proceeding, changes are taking place in the reaction mixture. Substrate is disappearing, the products of the reaction are being formed, and the forward reaction may be opposed by a reverse process. In some cases other factors too may be at work, such, for instance, as changes of pH due to the formation or utilization of acid or alkali. All enzymes are sensitive to changes of pH in their immediate environment, all enzymes are influenced by the concentration of substrate available to them, and many are actually inhibited by the products of their own activity. If, therefore, we wish to obtain a reliable measure of the activity of an enzyme under any given set of conditions, it will be necessary either to avoid these changes in the reaction mixture or else to make some suitable allowance for them.

Two main procedures are available. In the first place we can measure the *length of time required to produce a given amount of chemical change*. In this case the amount of substrate used up,

the amounts of products formed, the change of pH if any, and the extent of other changes likely to interfere with the enzyme will be the same in every experiment, so that different experiments will be comparable one with another, always provided that the enzyme is stable under the conditions selected. In such cases we can use time as a measure of the activity of the enzyme: actually, of course, the reciprocal of the time will be proportional to the activity of the catalyst, since an enzyme preparation that is half as active will take twice as long to produce the same amount of chemical change.

The second method is usually preferred, and consists in measuring the *amount of chemical change taking place over a very short interval of time from the start of the reaction*. Provided that the time interval can be made short enough, the changes in the composition of the reaction mixture will be small enough to be neglected. Ideally we should measure the *instantaneous initial velocity*, which is not a practicable proposition, but many excellent micro-methods are now available by the use of which we can obtain very good approximations to the instantaneous initial reaction velocity, and hence to the activity of an enzyme under any given set of conditions.

(ii) *The Influence of Temperature.* Most chemical reactions are influenced by temperature, the reaction velocity increasing with rising and decreasing with falling temperature. Enzyme-catalysed reactions are no exception to this general rule, but, because enzymes are very susceptible to thermal inactivation, the higher the temperature becomes, the more rapidly the catalytic properties of the enzyme are destroyed. For any given set of experimental conditions, therefore, it is possible to find what is called an *optimum temperature*, i.e. a temperature at which the greatest amount of chemical change is catalysed under that particular set of conditions. At suboptimal temperatures the enzyme is relatively more stable and therefore lasts longer, but the reaction which it catalyses proceeds more slowly. At temperatures above the optimum, on the other hand, the reaction takes place more rapidly but the catalyst is more rapidly destroyed.

There has been a good deal of misunderstanding on this subject in the past, for many biologists have supposed that the optimum temperature of an enzyme is a fixed and unalterable characteristic. A rise of temperature has a dual effect upon an enzyme-catalysed process: it increases the rate of the reaction, but it also increases the rate of thermal inactivation of the catalyst itself. Consequently, if we work over a period of a few seconds the

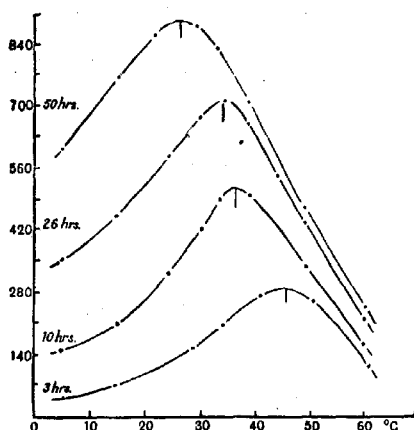


Fig. 2. Influence of temperature on digestive proteinase of *Telhyum*. Ordinate: mgm. amino-acid nitrogen per litre. (Substrate, gelatin: after Berril, 1929.)

optimum temperature may be very high indeed, because the catalytic properties of the enzyme do not need to be long lived. If, on the other hand, we choose to work over a period of a few days, a much lower optimum will be found since the enzyme must now last for a much longer period. It follows, therefore, that the time factor must be taken into account when we seek to determine the optimum temperature of any given enzyme, and that time and temperature are interdependent variables. The relationship between time and the optimum temperature of the digestive proteinase of an ascidian, *Telhyum*, is shown in Fig. 2.

With crude enzyme preparations such as were used in the earlier work on enzymes, the activity was usually of a rather low order, and it was therefore necessary to incubate the enzyme with its substrate for an hour or more in order to get a reasonable amount of chemical change. Under conditions of this kind most enzymes show an optimum temperature of about 30–40° C. This led to the suggestion that, when animals became homoiothermic, they settled on a body temperature of the order of 35° C. because their enzymes would 'work better' in that neighbourhood than at any other. But consider the case of *Tethyum*. Over a period of 2 hr. the optimum temperature of the digestive proteinase is of the order of 50° C., which is well above the thermal death-point of this species. *Tethyum* normally lives at temperatures in the neighbourhood of 15° C., and the digestion of its food takes about 50–60 hr. under natural conditions. If the optimum temperature is determined for a period of 55 hr. the value found is about 20° C., so that there is, after all, a nice adjustment of the enzyme to the biological requirements of the animal. This seems fairly generally the case, for 'there is evidence that the time taken for the passage of food through the gut at any normal temperature corresponds to the period which is optimal for enzymatic action at that temperature' (Yonge).

The thermal inactivation of enzymes is interesting from the physico-chemical viewpoint as well as from that of the biological behaviour of enzymes, for it yields important clues to the chemical nature of enzymes themselves. For most chemical reactions we find a temperature coefficient, represented by Q_{10} , of approximately 2; i.e. the rate of the reaction is approximately doubled for a rise of temperature of 10° C. If we determine the rate of thermal inactivation of enzymes in the neighbourhood of 70–80° C. we find values of the order of several hundreds. Temperature coefficients of this order are known for reactions of only two kinds; for the thermal inactivation of enzymes on the one hand, and for the thermal denaturation of proteins on the other. There is here, therefore, a striking indication that enzymes may be of protein nature, and that the process of thermal inactivation is analogous to, if it does not actually consist of, denaturation.

This latter point is stressed somewhat by the fact that enzymes are much less susceptible to thermal inactivation in the dry than they are in the wet state, while proteins appear to be more resistant to denaturation in the dry condition than they are in solution.

(iii) *The Influence of pH.* The catalytic powers of an enzyme are, as a rule, exercised only over a somewhat restricted range of pH. Within this range the activity passes through a maximum at some particular pH, known as the *optimum* pH, and then falls off again. Fig. 3 illustrates the activity/pH relationships of several enzymes.

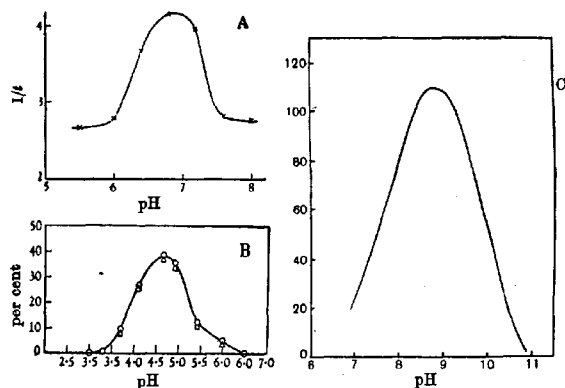


Fig. 3. Influence of pH on the activity of some enzymes.

A. *Salivary amylase* (substrate starch + NaCl). Ordinate: reciprocal of time taken to reach the achromic point. Results of a class experiment. B. *Papain-cysteine* (synthesis of carbobenzoxy-glycyl-anilide). Ordinate: yields as % of theoretical maximum, estimated by isolation (o) and titration (Δ). After Bergmann & Fraenkel-Conrat (1937). C. *D-Amino-acid oxidase* (substrate DL-alanine). Ordinate: oxygen uptake in 10 min. (μ l.). Results of Krebs (1935), after Green.

Generally speaking, the optimum pH is characteristic of a given enzyme, though under certain special conditions and in certain groups of enzymes the optimum pH may vary. This is true of the proteolytic enzymes, for example, and pepsin has an optimum pH that varies between 1.5 and 2.5 or thereabouts, different optima being found with different protein substrates.

A given carbohydrase, on the other hand, shows always the same optimum pH, even when acting upon different substrates.

In its general form the pH/activity curve of a typical enzyme closely resembles that obtained by plotting the degree of ionization of a simple ampholyte such as glycine against pH. It will be recalled that most of the properties of solutions of ampholytes such as proteins and amino-acids—such properties as solubility, osmotic pressure, conductivity, viscosity and so on—pass through either a maximum or a minimum at some particular pH, the so-called isoelectric pH. These changes are attributable to changes in the ionic condition of the ampholytes themselves. Being a zwitterion, any given protein, for example, can exist in a number of different ionic forms, and one of these, the isoelectric form, possesses a number of special and peculiar properties. It is therefore tempting to suggest that an enzyme may be regarded as a protein, and that of all the ionic forms in which it can therefore exist, only one particular ionic species possesses catalytic properties, this being the species which preponderates at the optimum pH. This may or may not be identical with the isoelectric form, and we shall have a little more evidence on this point later on (p. 50).

A further indication of the proteinaceous nature of enzymes is that extremes of acidity and alkalinity, which lead to an irreversible denaturation of proteins, lead also to the inactivation of the majority of enzymes. Moreover, these are irreversible changes, unlike those which are observed in the immediate vicinity of the optimum pH and which are for the most part reversible.

Generally speaking, enzymes are most stable in the neighbourhood of the optimum pH so that the observed optimum pH does not vary with time. The optimum pH of an enzyme is therefore a more characteristic feature than its optimum temperature. But if, as is sometimes the case, the enzyme is one which is very unstable at or near its pH optimum, the value observed will, of course, vary with the duration of the experiment. Accurate determinations of the optimum pH can only be made in such cases by working over very short intervals of

time. In the case of arginase, for example, the optimum pH is about 7-8 for a period of an hour or so, but the true optimum lies at about 10, a pH at which arginase is very unstable indeed. The case of arginase, however, is an unusual one: most enzymes have their pH optimum not very far from neutrality, most commonly between pH 5 and 7. Pepsin, however, has its optimum at an unusually acid value of 1.5-2.5, the precise figure depending upon the identity of its substrate.

(iv) *The Influence of Protein Precipitants.* Enzymes are inhibited by many different groups of chemical reagents, as well as by such physical factors as high temperatures, violent mechanical agitation, ultra-violet radiation and so on, all of which lead to the denaturation of proteins. Many protein precipitants also lead to the inactivation of enzymes. In this section special attention may be drawn to the effects of two groups of enzyme inhibitors which act also as precipitants for proteins, viz. the salts of heavy metals on the one hand and, on the other, the so-called 'alkaloidal reagents'. The former precipitate proteins by virtue of the heavy, positively charged ions to which they give rise in solution, and the alkaloidal reagents, which include such substances as trichloroacetic acid, tannic acid and phosphotungstic acid, act by virtue of their heavy, negatively charged ions. That all these agents are powerful inhibitors of enzymic activity strongly suggests that enzymes are proteinaceous in composition.

More precise indications to the same effect are to be had by studying the effects of small concentrations of inhibitors of this kind. If it is indeed true that enzymes are proteins, we should expect to find that, in common with the proteins, they will be positively charged in acid solutions and therefore susceptible to the action of the negatively charged ions of phosphotungstic acid, for example. In alkaline solutions, on the other hand, they would be expected to be negatively charged and susceptible therefore to the action of positively charged ions, for example, Ag^+ . This problem has been carefully investigated for a few enzymes, and the results obtained in the case of yeast saccharase are shown in Fig. 4. It will be seen that the effects of gradually

increasing concentrations of silver ions are most marked on the alkaline side of the optimum pH. Phosphotungstic acid produces similar effects on the acid side.

These results show that the behaviour of yeast saccharase with respect to these inhibitors is consistent with the view that this enzyme is a protein. In practice, however, the concentration of

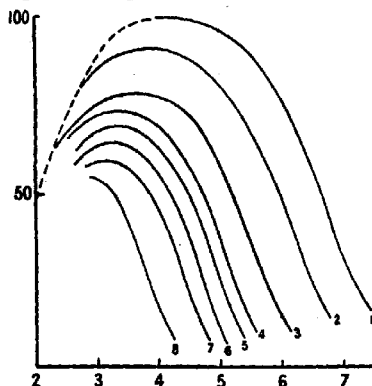


Fig. 4. Influence of small, increasing concentrations of silver ions on activity of yeast saccharase. Ordinate: initial velocities of hydrolysis of sucrose. Abscissa: pH. (After Haldane, from Myrbäck, 1926.)

Curve	Conc. Ag^+	Curve	Conc. Ag^+
1	0	5	$4 \times 10^{-4} \text{ M}$
2	$5 \times 10^{-7} \text{ M}$	6	10^{-4} M
3	10^{-6} M	7	$2 \times 10^{-4} \text{ M}$
4	$2 \times 10^{-6} \text{ M}$	8	10^{-4} M

silver ions required to produce complete inhibition of yeast saccharase is much smaller than that needed actually to precipitate proteins, and this suggests that the effect of Ag^+ is not a general one upon *all* the negatively charged centres of the protein molecule, but a localized and very specific one upon particular centres which are responsible for the catalytic properties of the presumptive saccharase protein. There thus emerges the notion that enzymic activity is not a property of the protein molecule as a whole, but rather that it is associated with certain special 'active' groups or centres.

SUMMARY

1. Enzymes are complex, organic catalysts of high molecular weight, produced by living cells but capable of acting independently of the cells that produce them. They are characteristically thermolabile and highly specific.

2. Several kinds and degrees of catalytic specificity can be recognized. The majority of enzymes show stereochemical specificity, but, in addition, their specificity may be low or very high with reference to the chemical constitution of their substrates.

3. Enzymes are profoundly affected by many physical and chemical factors, and determinations of their activity therefore require to be made under very closely controlled conditions.

4. Enzymes are in all probability of protein nature. Every enzyme so far isolated has proved to be either a simple or a conjugated protein. The behaviour of enzymes towards heat, changes of pH and protein precipitants is consistent with the supposition that they consist of protein material.

CHAPTER II

THE NATURE OF THE CATALYTIC PROCESS

THE UNION OF THE ENZYME WITH ITS SUBSTRATE

It is difficult to imagine how a catalyst of any kind can influence the rate of a chemical reaction unless it actually participates in that reaction. Most authorities agree that catalysts do in some manner combine with the substance or substances upon which their catalytic influence is exerted, but there has been much difference of opinion as to whether the union is of a 'physical' or adsorptive kind, or whether it is to be regarded as 'chemical'. But it is difficult to maintain that there is any fundamental difference between these types of union: rather must they be regarded as two extremes of one and the same phenomenon. In so far as it is possible to distinguish between adsorption and chemical combination it may be said that adsorption is, on the whole, a less specific and more freely reversible process than chemical combination. Calcium carbonate is a good example of what we should call a chemical compound, formed by the chemical union of carbon dioxide and calcium oxide. Yet at high enough temperatures the product dissociates freely, as though, by raising the temperature, we had converted a chemical into an adsorptive union.

While it is true that adsorption is often relatively unspecific, there is evidence in plenty that it can be very specific indeed. Thus we find that a positively charged material such as magnesium oxide will adsorb negatively charged dyes like methylene blue from aqueous solution, but fails to take up a positively charged dye such as eosin. Similarly, a protein will take up negatively charged dyes in solutions acid to the isoelectric pH, in which it is positively charged, while on the alkaline side it takes up positively but not negatively charged dyes. At or very near the isoelectric pH it will usually take up a little of both,

since, being a zwitterion, it carries an equal number of positive and negative charges at one and the same pH.

Clearly, therefore, several factors have to be taken into account when we are considering adsorption. The nature of the surface at which the adsorption takes place is certainly of importance. Carotenoid pigments, for example, are adsorbed at a magnesium oxide/petrol ether interface but not at a magnesium oxide/alcohol interface. Charcoal can be used to adsorb coloured impurities of many kinds from aqueous solution, but is relatively useless in chloroform, and so on. The second important factor is, of course, the chemical nature of the material being adsorbed. It is not difficult to understand that a given surface may be so specialized, whether by virtue of its charge or for some other reason, as to be capable of taking up, i.e. reacting with, substances of one particular kind. Nor, if we allow for its possible topographical specialization, is it difficult to imagine that a particular surface may be capable of reacting with one particular substance and one only.

There is nothing inherently improbable in the idea that an enzyme actually unites with its substrate, and it is difficult, indeed, to imagine how the facts of specificity could otherwise be accounted for. Until recent years there has been no direct evidence that an enzyme enters into combination with its substrate, but studies of the kinetics of enzyme-catalysed reactions had already made it clear that the assumption of such a union is in fact warranted.

Keilin has provided direct evidence for the formation of an enzyme-substrate complex between peroxidase and hydrogen peroxide. If peroxidase is added to its substrate in the presence of a suitable hydrogen donor such as pyrogallol, a vigorous reaction ensues, in which the pyrogallol is oxidized and the hydrogen peroxide reduced. In the absence of any hydrogen donor, however, the hydrogen peroxide does not undergo reduction. Now peroxidase is an iron-porphyrin derivative and as such has a strong absorption spectrum, displaying four bands at 645, 583, 548 and 498 $m\mu$ respectively. If hydrogen peroxide is added to a strong solution of the enzyme there is a sharp

change in colour and the spectrum changes completely. Only two bands at 561 and 530.5 $m\mu$ respectively can now be seen. This can only mean that some kind of reaction has taken place between the enzyme and its substrate. Moreover, the amount of hydrogen peroxide required just to convert the whole of the enzyme into the new compound is equivalent to exactly one molecule of hydrogen peroxide for each atom of peroxidase-iron. Another compound, with bands at 583 and 545.5 $m\mu$, is formed when the amount of hydrogen peroxide is increased to about 100 molecules per atom of peroxidase-iron.

Somewhat similar observations have been made with catalase. If hydrogen peroxide is added to catalase a violent reaction takes place, the substrate being converted into water and molecular oxygen. If, however, the enzyme is first treated with sodium azide, which inhibits its activity, there is only a slow reaction when hydrogen peroxide is added. The catalase-azide complex has a strong absorption spectrum with bands at 624, 544 and 506.5 $m\mu$, changing on addition of hydrogen peroxide to a spectrum with two bands at 588 and 547 $m\mu$. The original spectrum reappears when all the hydrogen peroxide has been decomposed, but the addition of more substrate will then restore the two-banded spectrum. These observations show that the (inhibited) enzyme reacts in some way with its substrate.

INFLUENCE OF CONCENTRATIONS OF THE ENZYME AND ITS SUBSTRATE

The rate of any enzyme-catalysed process depends, other things being equal, upon the concentrations of the enzyme and of its substrate, and an examination of the effects of these and other factors is very important for any understanding of enzymic catalysis. In the vast majority of cases we find that, with a fixed quantity of enzyme, the reaction velocity increases with increasing substrate concentration until a limiting value is reached. Fig. 5 shows the results of a typical experiment carried out along these lines. The magnitude of the limiting velocity finally attained depends upon the concentration of the enzyme used and is, in

fact, proportional to that concentration. These observations can be accounted for in terms of the theory brought forward by Michaelis.

For the purposes of the argument it is assumed that the enzyme and its substrate react together in some way to form an unstable complex, which then breaks down to yield the reaction

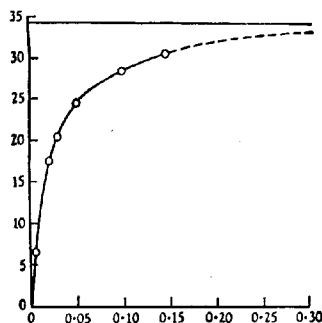
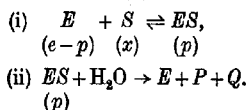


Fig. 5. Influence of substrate concentration on activity of yeast saccharase. Ordinate: initial velocity of hydrolysis. Abscissa: molar concentration of sucrose. (After Haldane, from Kuhn's data, 1923.)

products. If we choose a case such as the hydrolysis of sucrose by saccharase, these assumptions can be expressed in the following equations:



The enzyme is represented here by E , the substrate by S and the intermediate enzyme/substrate complex by ES , while P and Q are the products of the process.

If we represent the total enzyme concentration as e it follows that, since an amount p is bound up in the form of ES , the concentration of free enzyme will be equal to $(e - p)$. The reaction velocity, which we will call v , is the rate at which the products are formed, and this will clearly be proportional to the concentration, p , of the unstable complex ES . We are now in a position

to apply the principles of the mass law to our equations and in this way to make predictions which, if they prove to be in accordance with experimental observations, will provide evidence of the soundness of the assumptions epitomized in equations (i) and (ii).

It is necessary, before going further, to realize clearly that the concentrations represented by e , p , x and $(e-p)$ must, if we are to apply the mass law, be expressed as molecular and not as percentage concentrations. This fact is doubly important here because we are dealing with enzymes, which are colloidal materials, having very great molecular weights. Let us for the moment consider the enzyme E as a protein with the comparatively modest molecular weight of about 60,000, and compare it with a substrate such as urea with a molecular weight of 60. If we were to prepare 1% solutions of the pure enzyme and of the substrate, the molar concentration of the substrate solution would be no less than 1000 times that of the solution of enzyme. This point is of considerable theoretical importance, as we shall see, but it serves also to emphasize the relatively enormous activity of enzymes. They occur in living cells and tissues in minute amounts, amounts so small that the molecular concentrations are infinitesimal. Yet it is upon their catalytic activity that the life of the cells depends.

Returning now to Michaelis's theory we see that the following statements can be made:

$$\begin{aligned}\text{For equation (i) rate of forward reaction} &= x(e-p) k_1, \\ \text{rate of reverse reaction} &= p k_2,\end{aligned}$$

where k_1 and k_2 are the velocity constants of the forward and backward reactions respectively. Hence, when the system is in equilibrium,

$$x(e-p) k_1 = p k_2;$$

$$\text{therefore} \quad \frac{x(e-p)}{p} = \frac{k_2}{k_1} = K_m. \quad (\text{A})$$

Here K_m , the ratio of two constants, is itself a constant. This is called the Michaelis constant, and its particular significance will be considered later.

We are very seldom in a position to evaluate either e or p , since even if we had a perfectly pure enzyme at our disposal its molecular weight would probably be unknown. These terms, e and p , must therefore be eliminated from our equations, and this can be done through the following considerations. The reaction velocity v , for the decomposition of ES (equation (ii)) will be proportional to p and also to the concentration of water, but since the concentration of water in the system does not change appreciably we can write

$$v = kp, \quad (B)$$

where k is a constant. By combining this with equation (A) we could eliminate p , but the term e would still remain, and this, like p itself, we are usually unable to evaluate. But let us consider a special case in which there is a large excess of substrate. This case is not a fictional invention since, on account of the great disparity of molecular weight between E and S , x will usually be much greater than e . In the presence of a large excess of substrate, therefore, $[S]$ will be very much greater than $[E]$ so that virtually all the enzyme will be converted into ES , when $p = e$. Now we have just seen (equation (B)) that the reaction velocity is proportional to p , and in the presence of a large excess of substrate p attains the value of e . Consequently, in the presence of a large excess of the substrate, the reaction velocity will attain a limiting value which may be called V . We thus have, for this special case, a third equation:

$$V = ke. \quad (C)$$

Dividing (B) by (C) we get

$$\frac{v}{V} = \frac{p}{e}. \quad (D)$$

It is now possible to get rid of the unwanted terms from equation (A). We can rewrite (A) as follows:

$$x(e - p) = pK_m;$$

therefore

$$ex - px = pK_m,$$

$$ex = px + pK_m = p(x + K_m),$$

and

$$x = \frac{p}{e}(x + K_m).$$

Substituting for p/e from equation (D) we get the Michaelis equation:

$$x = \frac{v}{V} (x + K_m),$$

and hence

$$v = \frac{Vx}{x + K_m}.$$

This equation allows us to predict the manner in which the reaction velocity should be influenced by substrate concentration. It is, in fact, the equation of a rectangular hyperbola with the following properties (see Fig. 6):

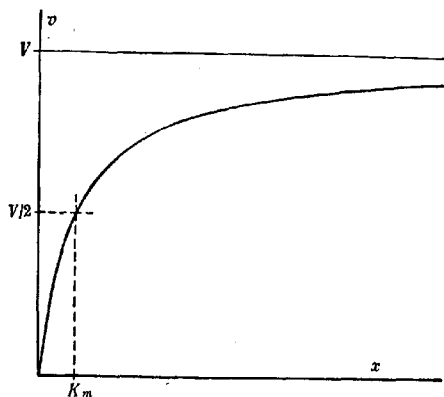


Fig. 6. Theoretical curve for Michaelis's equation,

$$v = \frac{Vx}{x + K_m},$$

where v = initial reaction velocity, x = concentration of substrate.

(a) the limiting velocity V , is the asymptotic value to which the reaction velocity tends as the concentration of the substrate is increased, and

(b) the Michaelis constant (K_m) corresponds to that substrate concentration at which half the limiting velocity is developed.

This fact is readily understood if we substitute $V/2$ for v in the Michaelis equation itself:

$$\frac{V}{2} = \frac{Vx}{x + K_m};$$

therefore

$$\frac{1}{2} = \frac{x}{x + K_m},$$

$$2x = x + K_m,$$

and

$$x = K_m,$$

when the reaction velocity is one-half the limiting velocity.

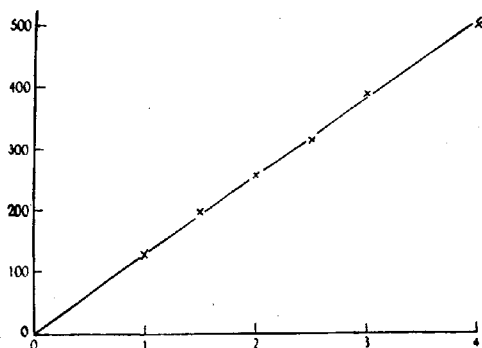


Fig. 7. Influence of enzyme concentration; yeast saccharase. Data from a class experiment. Ordinate: mg. invert sugar formed. Abcissa: ml. saccharase solution.

If the rectangular hyperbola of Fig. 6 is now compared with the curve of Fig. 5, which portrays the results of experimental observations, there can be no doubt that Michaelis's theory is in excellent agreement with the experimental results. Moreover, we have seen (equation (C)) that, according to this theory, the limiting velocity attained in the presence of an excess of substrate, should be proportional to the concentration of enzyme, and this also is in agreement with the results of experimental enquiry (see Fig. 7).

Atypical results are not infrequently obtained in experiments designed to test the validity of Michaelis's predictions, but these are usually due to interference by some factor or other, e.g. inhibition of the enzyme by the products of its own activity. When due allowance is made for this interference the corrected results are found to agree with theoretical prediction.

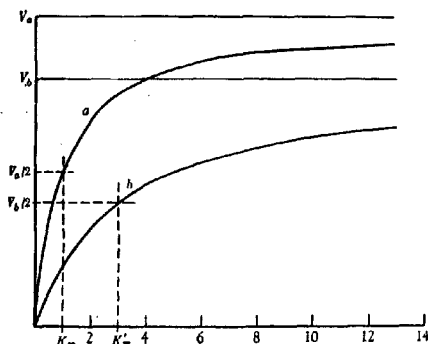


Fig. 8. Action of a group-specific enzyme upon two different substrates; for explanation see text. Reaction velocity in arbitrary units. Ordinate: reaction velocity. Abcissa: molarity of substrate.

The agreement between theoretical requirements and experimental observation goes far towards justifying the assumption upon which the theory was originally based, namely, that the enzyme actually combines with its substrate to form an unstable and correspondingly reactive complex. It may, of course, be argued that the same theoretical equation might be derived equally on the basis of different assumptions, but there is other and more direct evidence for the formation of enzyme/substrate compounds to which we have already referred (p. 29).

The Michaelis constant deserves a little further consideration. Let us suppose that we have an enzyme of low or of group specificity, and let us consider its activity towards two different substrates, *a* and *b*. If the relationships between reaction velocity and substrate concentration are experimentally determined we get a pair of hyperbolic curves like those of Fig. 8. For each of

the two substrates there is a K_m value, and in the figure it will be observed that for b the value (K'_m) is greater than for a (K_m). This means that in order to get the same velocity out of a given concentration of enzyme, b must be taken at a higher concentration than must a . This must mean that the enzyme has a smaller affinity for b than for a : in other words a high K_m is indicative of a low enzyme-substrate affinity, and vice versa. Thus, not only does Michaelis's theory furnish us with evidence that the production of an enzyme-substrate compound is an essential part of the catalytic process: it provides at the same time a means whereby the affinity of the enzyme for its substrate can be numerically evaluated.

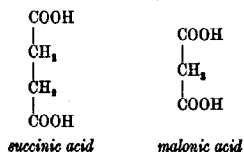
As we go on we shall see that the behaviour of enzymes is best explained on the supposition that they react with their respective substrates to form reactive complexes. In addition to the indirect evidence afforded by the applicability of Michaelis's theory there is a considerable mass of indirect evidence from other sources, as well as the recent direct evidence to which we have already referred.

COMPETITIVE INHIBITION

A great deal of information regarding the nature of enzymes and their mode of action has been gained by considering their inhibition, and studies of this kind have, as we have seen, done much to confirm the view that enzymes are made up essentially of protein material. Many enzymes are inhibited by the products of their own activity, and many more by substances which are structurally related to their substrates. In many such cases the inhibition is of what is known as the competitive type. A well-known case is found in the competitive inhibition of succinic dehydrogenase by malonate.

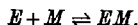
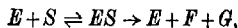
If we take succinate together with succinic dehydrogenase we have a system in which, under suitable conditions, it is easy enough to measure the reaction velocity in terms of the rate of oxidation of the succinate. If now malonate is added the rate of oxidation is promptly diminished, but increases again if more

succinate is added. Malonic acid is a dicarboxylic acid, the structure of which is closely related to that of succinic itself:



Malonate is able to combine with the enzyme, just as does the substrate, succinate. But whereas the enzyme-succinate complex breaks down to yield the reaction products, the enzyme-malonate complex contributes nothing to the reaction velocity. In consequence, a part of the enzyme is held in the form of enzyme-inhibitor complex, and so is not available for the catalysis of succinate oxidation, and the reaction velocity accordingly diminishes.

This system may be more precisely described in the following manner. We have the following equilibria and reactions to consider:



The rate of oxidation of the succinate is determined by $[ES]$, and this can be increased *either* by increasing $[S]$ or by decreasing $[M]$, which indicates that these two substances 'compete' for possession of the enzyme.

Now if the two compounds reacted at different points on the enzyme molecule there is no reason why they should not both be accommodated at the same time, each independently of the other. The fact that they do compete however shows that both unite with the enzyme molecule at precisely the same point.

Many other cases of the same kind are known. Thus yeast saccharase is competitively inhibited by fructose, and xanthine oxidase by adenine. That competitive inhibition exists at all is an indication that the substrate does not unite at arbitrary groups on the enzyme molecule, but *only at certain particular groups, and not elsewhere*. Thus these considerations not only

confirm the view that a union is set up between an enzyme and its substrate when the two are brought together: they go further, by showing that the union is very specific not only in nature but also in locality.

ACTIVATION OF THE SUBSTRATE

The combination of an enzyme with its substrate seems to be the fundamental and essential step in the catalytic process, for it is as a result of this union, presumably, that the substrate molecule becomes more chemically reactive than it was in the free, uncombined state, and is more easily split, oxidized, reduced or whatever the case may be. We refer to this increase in chemical reactivity by saying that the enzyme has 'activated' its substrate, or that the substrate has undergone 'activation'. We do not know what are the precise intramolecular changes that underlie activation, but we do know of other cases in which something of the sort takes place.

Let us consider the behaviour of haemoglobin. This compound consists of a protein, globin, to which is attached a complex, tetra-pyrrolic ring-structure containing an atom of ferrous iron. The special and peculiar property of haemoglobin is its ability to react reversibly with oxygen, taking it up when the partial pressure is high and giving it off again at low partial pressures. It is particularly noteworthy that this performance involves no change in the valency of the iron, a fact which is implied by speaking of the 'oxygenation' as opposed to the 'oxidation' of haemoglobin. The important point for our present argument is that haem, by itself, does not possess this property. It acquires this property when, and only when, it is combined with the appropriate protein, globin. Free haem is very insoluble in water, and reacts spontaneously with oxygen to undergo oxidation to the ferric compound, haematin. Globin, by combining with haem, confers upon it a large measure of solubility in water, together with the new-found property of reacting reversibly with oxygen, without at the same time undergoing any change in the valency of the iron.

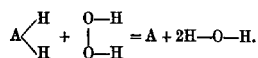
In addition to haemoglobin a number of other haem and haematin compounds with very special and peculiar properties are known, such, for example, as cytochrome, catalase and peroxidase. In each of these the haem or haematin system is present, but in none do we find the ability to combine reversibly with oxygen. The haem, presumably, is all right, but the protein is wrong. Thus the haem of haemoglobin possesses certain special properties which only become apparent when the haem nucleus is combined with the right kind of protein. While few, probably, would venture to assert that globin 'activates' haem, this case does show how the properties of a given substance can be profoundly and very specifically modified when the substance concerned enters into combination with the 'right' kind of protein.

We know too little about the phenomenon of activation to be able to make any clear picture of the changes which underlie it, but it is an interesting and highly significant fact that the hydrolytic processes catalysed by enzymes can as a rule be imitated by means of dilute acids, alkalis, or both, and sometimes merely by boiling water. Whether we treat sucrose, for example, with hot, dilute mineral acid or with saccharase prepared from yeast, we get precisely the same products, viz. glucose and fructose in equimolecular proportions. Saccharase, therefore, does not induce any new kind of reactivity in its substrate, but only exaggerates a tendency to react that is already inherent in the sucrose molecule.

ACTIVATORS AND COENZYMES

Activation of the substrate is an indispensable part of the chemical process catalysed by any enzyme, but it can take place without necessarily being followed by the hydrolysis, oxidation or other chemical modification of the substrate. Thus if we add peroxidase to a solution of hydrogen peroxide, the two unite to form an addition compound, as is shown by the resulting change in the absorption spectrum. But, in the absence of other materials, there the matter ends. If some substance capable of being oxidized is also added, AH_2 , say, there begins a rapid transference

of H atoms to the activated hydrogen peroxide so that AH_2 is oxidized and the peroxide reduced, thus:



This example suffices to show that, while activation is an essential part of the process of enzymic catalysis, activation only makes it possible for the reaction to take place: whether or not the reaction actually occurs may depend upon the presence of other materials, over and above the substrate and its activating protein, the enzyme. It is, in fact, true that in the vast majority of enzyme-catalysed reactions, substances other than the substrate and its activating enzyme-protein must also be present before any chemical change can be brought about. In processes of hydrolysis, for instance, water molecules form an indispensable part of the reaction system. The enzyme must therefore be considered as only a part, albeit the most important part from the biological point of view, of the whole reacting system. Similarly, in oxidation and reduction reactions, the majority of which are accomplished by the transference of pairs of hydrogen atoms from the substance being oxidized (the 'hydrogen donator') to the substance being reduced (the 'hydrogen acceptor'), we find that both substances must be present, together with the appropriate enzyme.

It has been known for many years that a considerable number of enzymes are unable to exert their catalytic influence except in the presence of certain appropriate materials which have become known as 'coenzymes' or 'activators'. It will be remembered that zymase, for instance, loses its activity if dialysed, and that this loss of activity is attributable to the removal from the juice of certain small, thermostable molecules in the absence of which fermentation cannot proceed. Recent work has shown that even such seemingly innocent substances as the ions of potassium, calcium, magnesium, chloride, phosphate and the like play indispensable parts in certain enzyme-catalysed processes. In such cases it is clear that the inability of the enzyme to act in the usual way might be due to one or other of two causes. Either

(a) the enzyme cannot activate its substrate because some accessory part of the enzyme itself has been removed, or (b) by contrast, the enzyme is capable of activating its substrate but no reaction takes place because some substance with which the substrate ordinarily reacts has been removed. It is possible to distinguish more or less sharply therefore between two groups of accessory substances, those which are parts of the activating system on the one hand, and on the other those which are a part of the reaction system but play no part in activation. Although it is difficult to justify any distinction in many cases, the tendency at the present time is to refer to accessory substances which are in effect a part of the activating system and are required before the enzyme can activate its substrate, as 'activators'. The term 'coenzyme', on the other hand, tends to be reserved for substances which play some part in the reaction catalysed by the enzyme, but not in the activation of the substrate. We shall follow this practice here. It must, however, be remembered that the *activation of an enzyme* by the appropriate 'activator' is quite distinct from the *activation of the substrate* that takes place as a result of its union with the specific activating protein or enzyme.

It is sometimes found that enzymes are secreted in a form in which they have no catalytic activity whatever, i.e. in the form of enzyme-precursors, or 'pro-enzymes'. The classical case to consider here is that of trypsinogen. The juice secreted by the pancreas of vertebrates contains a pro-enzyme, trypsinogen, which is devoid of action upon proteins. When the pancreatic juice enters the small intestine, trypsinogen is converted into the active proteolytic enzyme, trypsin. The change is attributed to the presence of an enzyme or enzyme-like factor present in the intestinal juice to which the name of enterokinase has been given. The change from trypsinogen to trypsin appears to be due to the removal from the pro-enzyme of a substance which acts, so to speak, as a 'mask' covering the reactive centres of trypsin itself. This 'mask' appears to be a polypeptide, and its removal is presumably due to proteolytic action on the part of enterokinase. This kind of activation, which for want of a better

term we may refer to as '*unmasking*', is now known to occur in several proteolytic enzymes, and will be dealt with at greater length when we come to consider them in more detail.

A second kind of activation, and one which is commonly encountered, might similarly be referred to as '*de-inhibition*'. Many enzymes are readily inhibited by mild oxidizing agents, and it frequently happens in the course of attempts to isolate a given enzyme that much of its activity is lost in the process as the result of oxidation by atmospheric oxygen, catalysed as a rule by traces of heavy metals present in the material, or derived from mincing machines or other metallic devices used in the preparation. In such cases the activity can very often be recovered by adding reducing agents such as cysteine or reduced glutathione. It has been shown by Hopkins that in the case of succinic dehydrogenase, though the same is not true of the lactic enzyme, any treatment tending to oxidize the $-SH$ groups of the enzyme-protein, so that $-S-S-$ cross-linkages are formed between adjacent molecules of the enzyme, results in the loss of dehydrogenase activity. These $-S-S-$ linkages can be reduced again by means of $-SH$ compounds, e.g. reduced glutathione, and the dehydrogenase activity of the enzyme returns therewith.

Activation by de-inhibition is a common process and may often be accomplished merely by removing inhibitory material. Cytochrome oxidase, an enzyme of central importance in respiratory metabolism, is powerfully inhibited by cyanide, but regains its activity if the cyanide is removed. Cytochrome oxidase is also powerfully inhibited by carbon monoxide in the dark, and a very striking case of activation by de-inhibition can be demonstrated in this case by exposing the preparation to strong light, which causes the carbon monoxide-oxidase compound to dissociate. Many other examples might be quoted.

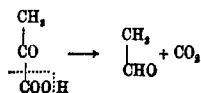
Activation by unmasking or de-inhibition is due, apparently, to the removal of material that inhibits by blocking the active parts of the enzyme. In other cases, however, it is less clear how the activator functions. Many enzymes concerned with phosphorylation, for example, require the presence of magnesium ions, but we do not know for certain what part these play. It

has been suggested that magnesium may furnish a means whereby the enzyme and its substrate can combine, i.e. that the enzyme-protein and the substrate react together through the magnesium ion. If this is the case, it follows that the enzyme-protein alone is unable to activate its substrate and that the magnesium must be regarded as a part of the activating machine. That this is so seems very probable, since one such enzyme, enolase, has been crystallized in the form of a magnesium-containing protein.

Another much-quoted case is that of salivary amylase and similar amylases. If a preparation of salivary amylase is dialysed it loses its power to digest starch at pH 6.8, which is the optimal value under normal conditions. Activity is regained by the addition of chloride ions, but the effect is not specific, and chloride may be replaced by other univalent or bivalent anions, though these are less effective (Fig. 9). Here, again, there is reason to suspect that the ionic activator is *a part of the activating machine*, and that in its absence the enzyme-protein cannot activate its substrate in the normal manner.

We have so far considered three main types of activators, those which act by unmasking the active groups of the enzyme, those which remove extraneous inhibitory material, and those which perhaps act because they are, in effect, a part of the enzyme. Other cases will be dealt with later when we consider individual enzymes in greater detail. We must turn now to consider accessory substances which confer activity upon inert systems because they *play a part in the chemical reaction* which follows upon the successful activation of the substrate. Substances of this kind are usually spoken of as coenzymes.

Of these the longest known, perhaps, is the co-carboxylase of yeast. Yeast, and the juice expressed from it by the Buchner technique, contain an enzyme known as carboxylase which, in the presence of co-carboxylase though not in its absence, catalyses the decomposition of pyruvic acid into acetaldehyde and carbon dioxide:



Co-carboxylase enters in some way into this reaction, which is called decarboxylation, though how it does so is still unknown. It is essential also for a more complex process known as oxidative decarboxylation, a reaction that takes place on a large scale in

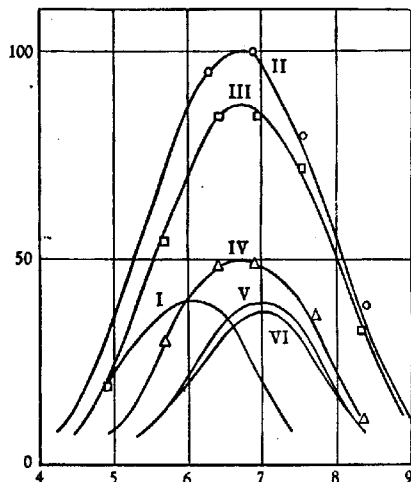
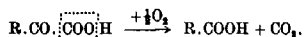


Fig. 9. Influence of anions upon activity of salivary amylase. Ordinate: initial velocity of hydrolysis. Abscissa: pH. (Substrate, soluble starch: after Myrback, 1926.)

Curve	Salt
I	Traces of NaCl
II	NaCl
III	NaBr
IV	KI
V	NaNO ₃
VI	KClO ₄

animal tissues and in which decarboxylation is attended by a simultaneous oxidative change:



This reaction, which is a good deal more complex than the 'straight' decarboxylation observed in yeast juice, is believed to be responsible for the production of the bulk of the carbon

dioxide formed in respiration, just as the straight decarboxylation catalysed by yeast juice is the source of the carbon dioxide produced in alcoholic fermentation. In both cases we find the same substance, co-carboxylase, as an essential part of the reacting system, and in both cases decarboxylation takes place. The coenzyme must therefore play some specific part in the decarboxylation reaction, though we do not at present know exactly what its role may be.

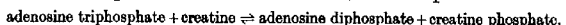
We are on surer ground when we consider the coenzymes involved in many oxidative processes. The majority of biological oxidations are carried out by the transference of hydrogen atoms from the substance undergoing oxidation, the 'hydrogen donator', to another substance, the 'hydrogen acceptor'. The dehydrogenases which catalyse reactions of this kind are not only specific to the hydrogen donator, but to the hydrogen acceptor as well. It follows, therefore, that in the absence of the appropriate hydrogen acceptor any given dehydrogenase, capable though it probably is of activating the hydrogen donator, cannot lead to any chemical change. This is true, for example, of the lactic dehydrogenase of muscle and of the alcohol dehydrogenase of yeast. In neither case does the substrate undergo oxidation unless the proper, i.e. specific, hydrogen acceptor is present. In these two systems the hydrogen acceptor is a substance known as coenzyme I or, less appropriately perhaps, as cozymase. This compound is able to take up a pair of hydrogen atoms from a suitably activated molecule of lactic acid or of alcohol, and the resulting reaction may be pictured as follows, e.g.



Of the dehydrogenases at present known, the majority require Co I as hydrogen acceptor and cannot use any other known, naturally-occurring substance in its place. Even the closely related Co II, which differs from Co I only in that it contains three instead of two phosphate radicals per molecule, cannot replace Co I. Nor can Co I replace the Co II which is required as hydrogen acceptor by a smaller group of dehydrogenases, of which hexosemonophosphate dehydrogenase may be cited as an example.

Co I and Co II are not by any means the only compounds which can act specifically as donators and acceptors of particular radicals or groupings. Indeed, during recent years, we have discovered numerous reactions in which particular radicals or groups are transferred from one molecule to another, and in every case it appears that a donator or acceptor substance is involved, over and above the enzyme which catalyses the transfer. These we need not discuss in any detail here since we shall refer to them frequently when we deal with intermediary metabolism, but a brief reference to one such system is desirable by way of example.

Muscle itself, and extracts prepared from it under suitable conditions, contain an important compound known as adenosine triphosphate. It also contains an important guanidine base, creatine. Under the influence of the muscle enzymes a phosphate radical can be transferred from adenosine triphosphate to creatine, and the process, which is reversible, can be represented thus:



The enzyme catalysing this transfer is specific both for the phosphate donator, adenosine triphosphate, and for the acceptor, creatine. But just as there are many dehydrogenases that can use Co I as a common hydrogen acceptor for different substrates, so too there are a number of phosphorylating enzymes which use adenosine triphosphate as a common phosphate donator with respect to their different substrates. We are probably justified, therefore, in regarding adenosine triphosphate as the coenzyme of all those phosphorylating enzymes which require its collaboration.

Co I and Co II, adenosine triphosphate and certain other compounds discharging comparable 'carrier' functions play a vital part in metabolism. Normally they occur only in very small concentrations in living tissues—Warburg and Christian, for example, could isolate only about 20 mg. of Co II from the red blood corpuscles of some 250 l. of horse blood—but the reactions in which they participate are very rapid indeed. Since these co-substances are essential for the occurrence of these reactions, and since they are present in such small amounts, it is clear that

we must regard them as true catalysts. Their catalytic influence is, in fact, no whit less important than that of the enzymes with which they collaborate.

PROSTHETIC GROUPS

In recent years a considerable number of enzymes have been greatly concentrated and finally obtained in crystalline form, and in many cases, notably among enzymes concerned with processes of oxidation and reduction, the molecule has been found to contain a non-protein moiety in addition to its protein component. Enzymes of this kind, therefore, are conjugated proteins, and the non-protein fragment is called the prosthetic group in each case.

The question arises whether or not there is any essential resemblance between the functional behaviour of a substrate, a coenzyme and a prosthetic group. In substances such as haemoglobin, haemocyanin and the like it has long been known that the non-protein part of the molecule is firmly attached to the protein component, and the special name of prosthetic group was coined to describe it. All the conjugated proteins, to which class haemoglobin and haemocyanin belong, were regarded as consisting of a protein portion to which there was firmly attached a prosthetic group of some kind. In more recent times we have made the acquaintance of conjugated proteins of which the prosthetic groups are relatively much more loosely attached. Thus there exists in the eggs of the lobster a green chromoprotein, ovoverdin, the prosthetic group of which can be removed by heating to about 60° C., but reunites with the protein on cooling. Still more striking, perhaps, are the visual chromoproteins, rhodopsin and porphyropsin, which dissociate on exposure to light, but reunite in the dark. Many such cases are now known, and the notion that a prosthetic group is necessarily firmly attached or screwed down to its protein partner has been abandoned.

If we import the same notions into the field of biological catalysis we find that, in the main, it is possible to distinguish

between substrates and coenzymes, which are only loosely and temporarily attached to the catalytic proteins with which they react, and prosthetic groups, which are relatively firmly fixed to their protein partners. The part played by the prosthetic group is precisely known in some cases. Certain oxidizing enzymes have a prosthetic group which functions as a 'built-in' hydrogen acceptor, taking over a pair of hydrogen atoms from the activated substrate and subsequently passing them on to another acceptor. In such cases the enzyme behaves as an activating protein and hydrogen acceptor rolled into one, and the functional behaviour of the prosthetic group in such a case is therefore analogous to that of the coenzyme of a typical dehydrogenase. The essential difference is that, whereas the partnership set up between the activating protein and the prosthetic group of an enzyme such as catalase is a relatively permanent affair, the partnership between, say, lactic dehydrogenase and lactic acid, or between the dehydrogenase and Co I, is only a temporary one on account of the relatively slight affinity between the partners.

The difference between coenzymes, substrates and prosthetic groups is therefore one of degree rather than of kind. Whether we consider Co I as a temporary prosthetic group of lactic dehydrogenase, or haematin as a permanent or built-in coenzyme of peroxidase, matters little so long as the functional significance of the various parts of the system is clear. What does matter is that we shall realize that the old, sharp distinction that seemed to exist between enzymes and carriers, and between substrates, coenzymes and prosthetic groups cannot now be justified, a fact which brings a new unifying influence to bear on our knowledge of biological catalysis.

QUANTITATIVE CHARACTERIZATION OF ENZYMES

Certain features of enzymic catalysis already alluded to in the first chapter of this book may now be considered in more detail. Enzymes in general may be considered under two headings, according as their substrates do or do not ionize. As examples of the former type we may consider the proteinases, and of the

latter type the carbohydrases. In all these cases the activity of the enzyme is profoundly affected by pH, and since the substrates of the carbohydrases, for example, do not ionize, the influence of pH upon these enzymes must be entirely due to its influence upon the catalytic proteins.

Knowing that enzymes are proteins, we may infer that they carry numerous ionizable groups, the ionic state of which depends upon the pH of the surrounding medium. Since there is some particular pH at which the enzyme is more active than at any other, we may suppose that, of all the possible ionic forms in which the enzyme-protein can exist, only one possesses catalytic properties, and that it is this form that predominates at the optimum pH. Michaelis and Davidson suggested that, since a change of pH in either direction away from the optimum leads to a diminution of catalytic activity, two kinds of groups must be involved in determining activity, the one kind being acidic and the other basic in nature. The enzyme, like any other protein, must be considered as an ampholyte, and in view of the close resemblance that exists between the dissociation curve of a simple ampholyte such as alanine on the one hand, and the pH/activity curve of a typical enzyme on the other, Michaelis and Davidson went on to suggest that the two halves of the pH/activity curve must correspond to the dissociation curves of the two particular groups or sets of groups upon the ionic condition of which the catalytic activity of the protein depends. For any given enzyme, therefore, the form and position of the pH/activity curve should be constant, even if the enzyme acts upon several different substrates, always provided that the substrates themselves do not ionize. This seems generally to be true.

If, therefore, we determine the pK values for the dissociation of the two sets of ionizable groups, which we can do by carefully plotting the pH/activity curve, we shall have determined in quantitative terms two constants that are characteristic of the enzyme. This has been done in only a few cases. It is considerably easier to determine the resultant of the two dissociation curves, i.e. the optimum pH; a number of pH optima for various enzymes are listed in Table 1.

TABLE I. pH OPTIMA OF SOME ENZYMES
(From Haldane's Tables)

Enzyme	Source	Substrate	Optimum pH
Pepsin	Stomach	Various proteins	1.5-2.5
Trypsin	Pancreas		8-11
Amylase	Saliva	Starch (+chloride)	6.7-6.8
"	Pancreas	"	6.7-6.8
"	Malt	Starch	5.2
α -Glucosidase	Gut	Maltose	6.1
"	Yeast	"	6.6
"	"	α -Methylglucoside	6.2
β -Glucosidase	Almond	Various β -glucosides	4.1-4.5
"	Malt	Cellobiose	5.1
Saccharase	Gut	Sucrose	6.2
"	Yeast	"	4.6-5.0
Lipase	Liver	Ethyl butyrate	8.3
"	Pancreas	"	7
"	<i>Ricinus</i>	Tributyrin	5
Succinic dehydrogenase	Muscle	Succinate	9
"	<i>Bact. coli</i>	"	8-10
Xanthine oxidase	Milk	Xanthine	5.5-8.5
Arginase	Liver	L-Arginine	9.8
Carboxylase	Yeast	Pyruvate	4.8
D-Amino-acid oxidase	Liver; kidney	DL-Alanine	9

In the case of enzymes that act upon ionizable substrates, the position is complicated by the fact that changes of pH will influence the ionic conditions both of the enzyme and of its substrate. Further, if we change the substrate, the shape and position of the pH/activity curve will be expected to change, and we do in fact find that enzymes such as pepsin and trypsin show different pH optima when acting upon different proteins. Nevertheless, if we stipulate some particular substrate in any particular case we can determine the optimum pH or the two pK values for that particular enzyme/substrate pair.

An interesting example of the usefulness of these ideas is found in connexion with the effect of chloride ions upon the activity of salivary and pancreatic amylases. If chlorides are removed, the more alkaline of the two pK values shifts from about 8 to 6.7, so that the pH/activity curve, which is the resultant of the two dissociation curves, becomes shifted over towards the left and loses height at the same time (see Fig. 9, p. 45). Presumably, therefore, one of the active groups of the enzyme ionizes differently according as chloride is or is not present.

Another characteristic property of enzymes that can be measured and expressed in quantitative terms is the Michaelis constant K_m . This, it will be remembered, is that concentration of substrate at which, in the presence of a given amount of enzyme, the reaction velocity attains half its limiting value. A list of some K_m values is given in Table 2. The Michaelis

TABLE 2. MICHAELIS CONSTANTS OF SOME ENZYMES
(From Haldane's Tables)

Enzyme	Source	Substrate	K_m
Pepsin	Stomach	Egg albumin	4.5%
Trypsin	Pancreas	Casein	2%
Amylase	Saliva	Starch (+ chloride)	0.4%
	Pancreas	"	0.25%
α -Glucosidase	Yeast	α -Methylglucoside	0.037-0.075 M
"	"	α -Phenylglucoside	0.021-0.050 M
β -Glucosidase	Almond	β -Methylglucoside	0.060-1.12 M
"	"	β -Phenylglucoside	0.040-0.065 M
Saccharase	Yeast	Sucrose	0.016-0.04 M
"	"	Raffinose	0.24-0.66 M
"	Gut	Sucrose	0.02 M
Lipase	Pancreas	Ethyl butyrate	>0.03 M
"	Liver	Ethyl- <i>d</i> -mandelate	0.0007 M
"	"	Ethyl- <i>l</i> -mandelate	0.0017 M
Succinic dehydrogenase	Muscle	Succinate	0.001 M
Xanthine oxidase	Milk	Xanthine; hypoxanthine	$<3 \times 10^{-4}$ M
"	"	Acetaldehyde	>1 M
D-Amino-acid oxidase	Liver; kidney	DL-Alanine	5×10^{-3} M
Carboxylase	Yeast	Pyruvate	0.01 M
Catalase	Liver	Hydrogen peroxide	0.025 M

constant differs from enzyme to enzyme, and varies also from substrate to substrate when the enzyme's specificity is not absolute. If we have an enzyme preparation that acts upon several glycosides, for example, we commonly find a different K_m for each substrate, but this does not tell us whether two or more different enzymes are concerned, or whether a single enzyme of group-specificity is at work. Preparations of yeast saccharase, for example, act upon both sucrose and raffinose, and the question arises whether yeast contains a raffinase as well as a saccharase. The K_m value for sucrose is about one-sixteenth as great as that for raffinose, but the ratio is always the same, no matter how the enzyme preparation may be prepared or purified. If two enzymes were concerned we should expect them to be

present in different proportions in different preparations made by different procedures, and it therefore follows that probably only one enzyme is concerned, a conclusion which is confirmed by the fact that the pH/activity curve has the same pK values and the same pH optimum whether sucrose or raffinose is the substrate.

A fourth characteristic constant can be determined in certain cases. It will be remembered that in the presence of a large excess of substrate, the reaction velocity of an enzyme-catalysed reaction reaches a limiting value V . If the concentration of the enzyme is known and is equal to e , then

$$V = k \cdot e,$$

where k is the velocity constant of the reaction. In general we cannot evaluate e , since the molecular weight of the enzyme must be known if this is to be calculated and, moreover, the enzyme must be available in a chemically pure form. These requirements are seldom fulfilled. Nevertheless, the value of k would be characteristic for a given enzyme acting upon a given substrate if we could but determine it. The best we can do in most cases is to determine V in the presence of an arbitrarily defined concentration of the enzyme. If the enzyme is one that works on more than one substrate, say on two compounds a and b , the ratio V_a/V_b will be constant if the enzyme concentration is the same in both cases. Even this second-best determination has proved itself valuable in deciding the identity of pairs of enzymes. Consider once more the case of yeast saccharase. This enzyme attacks sucrose twice as fast as raffinose, and the ratio remains the same from one preparation to another. If two enzymes were concerned we should expect the ratio to vary from case to case, but this does not happen, thus adding still more evidence of the identity of the presumptive raffinase with saccharase.

To establish the identity of one enzyme with another, even in relatively crude extracts, a number of ways are thus open. We can see whether the two behave in the same manner with respect to inhibitors and activators, and we can find out whether their

specificities are similar or different. These, however, are properties that cannot be exactly defined or numerically expressed, but further evidence of a perfectly quantitative nature can be obtained by measuring (a) the two pK values, given by the positions of the points of inflexion of the pH/activity curve, or in default of these, the optimum pH. In addition (b) the Michaelis constant may be determined for one or more different substrates, and (c) we may determine the limiting velocities corresponding to known or arbitrarily defined concentrations of the two enzymes.

SUMMARY

Summarizing our conclusions as to the nature of the enzyme-substrate union it may be said first of all that there is every reason to believe that such a union does in fact take place. The union is a very specific one; a given enzyme can combine with and activate only a very limited number of substrates and, often enough, only one substrate. There is reason to think that the reaction takes place at certain definite points on the surface of the enzyme, rather than at any arbitrary point or points, and it seems that the specificity of an enzyme is really a measure of the extent to which the enzyme and the substrate 'fit' at the points through which they unite.

Even when the activating protein fits the substrate well enough to allow union to take place between them, the 'fit' may be thought to be still slightly imperfect, so that the substrate molecule is subjected to some kind of internal strain which results in the increased chemical reactivity to which we refer as activation.

The essential function of an enzyme is that of activating its substrate. It may lose the power to do this for any of a large number of reasons, but in many cases the lost activity can be recovered. But even when activation has been accomplished, the substrate does not necessarily undergo any chemical change, since substances other than the substrate and its activating protein may also be required. We have considered ways in which

the power of activation may be restored to an enzyme that has lost it, and we have considered some cases in which accessory substances or coenzymes enter into the reactions taking place after the substrate has been successfully activated.

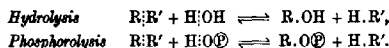
Finally, we have seen that it is possible to obtain quantitative data which are characteristic of individual enzymes.

CHAPTER III

HYDROLASES AND PHOSPHORYLASES

GENERAL INTRODUCTION

Of the splitting enzymes the *hydrolases*, which catalyse a hydrolytic splitting of their substrates, are the most numerous and widely distributed. A smaller but exceedingly important group of *phosphorylases* has been discovered in recent years, and these catalyse splitting, not by means of water, but by phosphoric acid:



(Here the symbol $\textcircled{\text{P}}$ represents $-\text{PO}_3\text{H}_2$, and this abbreviation will be frequently used throughout this book.) All digestive enzymes fall into the hydrolytic class, whether they are secreted or whether they discharge their functions intracellularly, but in addition to these we have to consider intracellular hydrolases and phosphorylases, the functions of which are not digestive.

Animal and plant tissues as a whole are known to contain intracellular enzymes which can be extracted and shown to catalyse the breakdown of proteins and other high-molecular materials into simpler units. It seems improbable that this is their sole function in the cell. After death, these enzymes do in fact lead to the digestion of much of the tissue substance, a process known as autolysis, and this is why game and certain kinds of meat are allowed to 'hang' before being cooked. This same process of autolysis is the first stage in the decomposition of dead organisms; bacteria and the biblical worms come on to the scene considerably later.

We have good reason to believe that the tissue constituents themselves are not permanent structures, but rather that they are constantly in a state of breakdown balanced by synthesis. This is true even of hard structures such as the bones and the teeth. It seems likely, therefore, that the intracellular enzymes must be concerned with catalysing both the breakdown and the

synthesis of the proteins, fats and carbohydrates of the cell. On theoretical grounds we must believe that an enzyme which can catalyse the degradation of its substrate must also be capable of catalysing the recombination of the products, and in a number of cases, e.g. among the lipases, such a reversibility can readily be demonstrated. In many other cases, however, attempts to demonstrate the catalysis of synthesis by an enzyme known to catalyse the corresponding breakdown have yielded only negative results. It must, however, be remembered that in order that a given chemical reaction may take place it is not enough that the reactants shall be in a reactive state: *the energy conditions also must be favourable*. Many hydrolytic reactions, e.g. the digestive hydrolysis of glycogen, appear to be virtually irreversible, apparently because energy is needed for the reversal but cannot be directly supplied. The intracellular degradation and resynthesis of glycogen proceed freely, however, but *by other pathways and through different intermediates* from those involved in digestion. Glycogen, which yields maltose as the end-product of its digestive hydrolysis, is not synthesized directly from maltose nor even from free glucose, but from α -glucose-1-phosphate, and the first product of its intracellular degradation is likewise α -glucose-1-phosphate. The latter can yield glycogen because the free energy-levels of both substances are practically identical, and significantly higher than that of free glucose, which must be phosphorylated before it can give rise to glycogen. By undergoing phosphorylation the free sugar is *raised to a higher energy-level* (see pp. 72-5).

The function of many of the intracellular enzymes must be regarded as that of maintaining an equilibrium between the complex materials of the cell substances and their simpler constituents, so that a balance is struck between synthesis and degradation. And we must not think of the equilibrium thus set up and maintained as in any sense a static affair, but as an essentially dynamic system, in which breakdown and resynthesis are proceeding simultaneously at high but equal velocity. We should do well to recall Hopkins's celebrated aphorism, that life is a dynamic equilibrium in a polyphasic system.

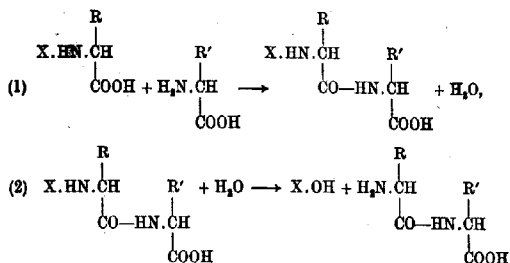
In what follows we shall consider the properties of a number

of the most widely distributed hydrolytic and phosphorolytic enzymes. If the next few pages seem to savour somewhat of the catalogue it is because we must necessarily know a good deal about individual enzymes before we can attempt to see how, in the living cell, tissue or organism, they are organized into the catalytic systems that underlie the metabolic processes inseparable from life itself.

PROTEASES

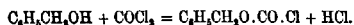
Until comparatively recently it was usual to distinguish between two main groups of enzymes concerned with the hydrolysis and presumptive synthesis of proteins and their breakdown products. On the one hand were enzymes such as pepsin and trypsin, which were believed to act upon proteins but not on smaller molecules such as those of the peptones and peptides. On the other hand was a group of so-called peptidases, known collectively as erepsin, and these were regarded as being devoid of action upon any but the relatively small molecules of polypeptides and, perhaps, peptones. Most of our earlier views have now been seriously modified or even abandoned. As is well known, the chemical synthesis of peptides was, until about 1932, a difficult undertaking, and of the enormous variety of possible peptides a mere handful was obtainable by synthetic chemistry. Our knowledge of the specificity of protein- and peptide-splitting enzymes was consequently fragmentary. In recent years, thanks mainly to the ingenious method introduced by Bergmann, peptides of many kinds hitherto unavailable have been produced and, in the meantime, a number of the enzymes themselves have been obtained in highly purified, crystalline form.

The older methods for the synthesis of peptides mostly involved covering the $-\text{NH}_2$ group of one amino-acid and condensing the protected product with a second amino-acid or its ester. A 'covered' dipeptide could thus be obtained from which, by removal of the covering group, the free dipeptide could theoretically be regenerated. The reactions involved may be written as follows, if we represent the covering group by X:

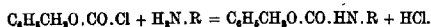


Tri- and higher peptides could be prepared by further condensations on to the free —COOH of the covered dipeptide formed in reaction (1) before removing the covering group. The use of the benzoyl radical, introduced by Curtius, made possible the synthetic production of numerous benzoylated peptides, but attempts to remove the benzoyl group by hydrolysis resulted in the simultaneous hydrolysis of the peptide bonds, so that the yields of free peptides were negligible at best. The use of other substituent radicals had little better success, and numerous other methods of synthesis were tried.

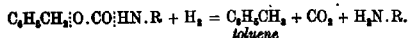
In Bergmann's method the covering group employed is one which can be removed by reduction, a treatment which does not at the same time open peptide links. Bergmann employs benzyloxycarbonyl chloride. This reagent is made by treating benzyl alcohol with phosgene in solution in toluene:



It reacts readily with the amino-group of an amino-acid to yield a *carbobenzoxy-derivative*, thus:



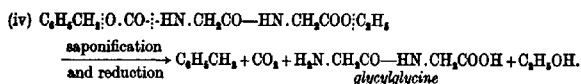
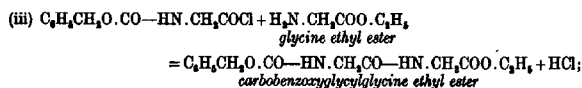
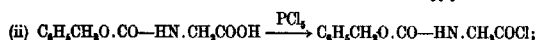
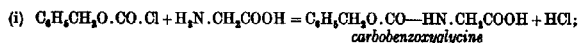
Its subsequent removal is accomplished by catalytic reduction with hydrogen in the presence of colloidal palladium:



These carbobenzoxy-compounds are very stable, and can readily be converted into the corresponding acyl chlorides so as to facilitate condensation with a second amino-acid. There is,

moreover, no racemization of the product under Bergmann's conditions.

As an example of a synthesis carried out by Bergmann's method we may take the relatively simple case of the preparation of glycylglycine. The reactions used are as follows:



By taking suitable precautions it is possible to prepare peptides containing dicarboxylic and dibasic amino-acids as well as the mono-amino-mono-carboxylic acids by this method, and with its aid peptides of many different kinds have now been made available for studies of the specificities of proteolytic enzymes.

Digestive Peptidases. The proteolytic enzymes of vertebrates fall into two groups, the first of which is mainly concerned with the degradation of the large molecules of the food proteins to yield smaller fragments, the second group completing the process initiated by the first and leading eventually to the liberation of free amino-acids. The first group includes *pepsin*, which arises from the gastric juice, and *trypsin* and *chymotrypsin*, formed from precursors present in the pancreatic juice. In the second group we have *carboxypeptidase*, contributed by the pancreatic juice, together with *aminopeptidase* and *dipeptidase*, which are present in the intestinal secretions. The proteolytic ('peptolytic') enzymes of the intestinal juice were formerly regarded as one enzyme, to which the name *erepsin* was given, but it is now known that erepsin is, in fact, a mixture of enzymes, each member of which is very much more specific than the original complex.

Activation

Four of these enzymes are actually secreted in the form of enzymatically inactive precursors which undergo activation by unmasking. Pepsin is secreted by the gastric mucosa in the form of *pepsinogen*, which is activated in the first instance by the hydrochloric acid of the gastric juice to yield pepsin itself. Pepsin, once formed, is capable of activating more pepsinogen, so that, once begun, the activation of pepsinogen is an autocatalytic process. Both pepsinogen and pepsin itself have been obtained in pure, crystalline form, and it has been shown that the conversion of the inert pro-enzyme into the active form is attended by a fall in molecular weight from some 42,000 to 38,000. A polypeptide of molecular weight 5000 or thereabouts is split off, and may be regarded as the 'masking' substance.

Trypsin and chymotrypsin also are secreted in the form of enzymatically inert precursors, *trypsinogen* and *chymotrypsinogen*. All four compounds have been crystallized. Trypsinogen is activated by an enzyme-like substance called enterokinase, which is present in the intestinal secretions and of which the precise nature is not yet known. Enterokinase acts upon trypsinogen to produce trypsin, which then activates more trypsinogen so that, as in the case of pepsinogen, activation is an autocatalytic process. In this case, however, there is no detectable change in molecular weight.

Chymotrypsinogen differs from trypsinogen in that it is not activated by enterokinase. It is, however, activated by trypsin. Chymotrypsin does not activate chymotrypsinogen, and in this case, therefore, activation is not autocatalytic. The activation of the enzyme precursors of pancreatic juice is thus started off by enterokinase, which activates trypsinogen with production of trypsin. The trypsin then activates more trypsinogen and chymotrypsinogen as well. The proteolytic enzymes of pancreatic juice do not therefore become active until they reach the small intestine and come into contact there with enterokinase.

Of the other peptidases only carboxypeptidase requires activation: the pro-enzyme is activated by trypsin, but not by enterokinase or chymotrypsin. Aminopeptidase and dipeptidase are

not activated in this way. They lose their activity if dialysed, but activity is restored by the addition of traces of manganese, which appears to be the natural activator for these enzymes.

Specificity

Biologically speaking it is possible to draw some sort of distinction between pepsin and the trypsins on the one hand—the proteinases of the old nomenclature—and the group of peptidases on the other. The digestion of the food proteins is begun by the ‘proteinases’, and the fragmentary products thus formed are further degraded by the ‘peptidases’ to yield in the end free amino-acids. It was formerly believed that pepsin and the trypsins are able to attack only large molecules, of the same order of size as the protein molecules of the food, and that the peptidases are only able to deal with molecules of the order of size found among polypeptides and perhaps peptones. More recent work, which became possible only when Bergmann’s method had made a wide variety of synthetic peptides available, has shown that pepsin, chymotrypsin and trypsin, as well as the peptidases, are able to act upon comparatively simple peptides, always provided that peptide linkages of the right kind are present. Bergmann therefore calls them all peptidases, but it is still possible to maintain a distinction between the two groups. The peptidases of the older nomenclature are able to split only those peptide links which join terminal amino-acid residues to the main chain. Pepsin and the trypsins, on the other hand, can act also upon peptide bonds remote from the terminal units and are accordingly called *endopeptidases*, by contrast with the *exopeptidases*, i.e. carboxy-, amino- and dipeptidases. Briefly, then, pepsin, trypsin and chymotrypsin, the ‘proteinases’ of the older nomenclature, become the *endopeptidases* of Bergmann’s nomenclature, while carboxypeptidase, aminopeptidase and dipeptidase, the ‘peptidases’ of earlier years, become the *exo-peptidases*.

Our ideas about the specificity of these enzymes have undergone a drastic change in recent years. It was formerly held that pepsin, trypsin and chymotrypsin are able to attack peptide

bonds at more or less any peptide linkage in the chain. But if both pepsin and trypsin are allowed to act upon the same protein we find that both enzymes together open up more peptide linkages than either alone, and it follows that both enzymes do not act upon the same, but upon different linkages. Thanks to the brilliant work of Bergmann and his colleagues we now know a good deal about the nature of the particular bonds attacked by the peptidases. One general fact may be emphasized at once: with certain exceptions, the peptidases as a whole act only upon normal peptide links, i.e. links formed between the α -amino- and α -carboxyl radicals of L-amino-acids. Enzymes capable of attacking peptides containing amino-acids of the D-series have recently been described, but these will not be considered here.

Pepsin can act only on peptide bonds of certain definite types. As Bergmann has shown, it can attack a peptide link lying between an L-dicarboxylic and an L-aromatic amino-acid, given certain conditions. These are, first, that the second carboxyl radical of the dicarboxylic acid residue must be free, and secondly, that there must not be a free amino-group in the immediate vicinity of the peptide linkage. Thus pepsin attacks carbobenzoxy-L-glutamyl- β -L-tyrosine, glycyl-L-glutamyl- β -L-tyrosine, carbobenzoxyglycyl-L-glutamyl- β -L-tyrosine and carbobenzoxy-L-glutamyl- β -L-phenylalanine. The influence of the free γ -carboxyl group of the glutamic acid residue is neutralized if there is a free amino-group nearby, for L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-tyrosine amide are resistant to pepsin. The resistance of carbobenzoxy-L-glutamyl-L-tyrosine amide is not due solely to the fact that the α -carboxyl group of the tyrosine is covered, for carbobenzoxy-L-glutamyl- β -L-tyrosyl-glycine is attacked, though more slowly than carbobenzoxy-L-glutamyl- β -L-tyrosine. Replacement of the L-acids by their D-isomers makes the peptides resistant to pepsin, for carbobenzoxy-D-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-D-phenylalanine are not attacked. These results are summarized in Table 3.

Chymotrypsin resembles pepsin in attacking peptide links in which aromatic amino-acids are involved, but whereas pepsin

attacks on the amino side of the aromatic acid, chymotrypsin acts on the carboxyl side. Thus both enzymes attack carbobenzoxy-L-glutamyl- β -L-tyrosyl- β -glycine amide, but do so at different points, as follows:

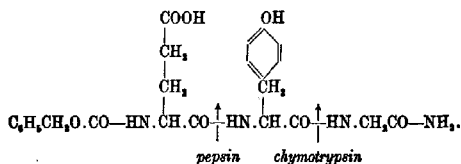


TABLE 3. ACTION OF PEPSIN UPON SYNTHETIC PEPTIDES

Substrate	Action of pepsin
Carbobenzoxy-L-glutamyl- β -L-tyrosine	+
Glycyl-L-glutamyl- β -L-tyrosine	+
Carbobenzoylglycyl-L-glutamyl- β -L-tyrosine	+
Carbobenzoxy-L-glutamyl- β -L-phenylalanine	+
L-Glutamyl-L-tyrosine	-
Carbobenzoxy-L-glutamyl-L-tyrosine amide	-
Carbobenzoxy-L-glutamyl- β -L-tyrosylglycine	+
Carbobenzoxy-D-glutamyl-L-tyrosine	-
Carbobenzoxy-L-glutamyl-D-phenylalanine	-

Chymotrypsin also attacks carbobenzoxy-L-tyrosyl- β -glycine amide and carbobenzoxy-L-phenylalanyl- β -glycine amide, for example, but its action is prevented by the presence of a free carboxyl radical in the immediate vicinity of the peptide link. Thus carbobenzoxy-L-glutamyl-L-tyrosyl-glycine, unlike its amide, is resistant to chymotrypsin, though acted upon by pepsin. On the other hand, L-glutamyl-L-tyrosyl- β -glycine amide is attacked by chymotrypsin and not by pepsin, since the effect of the free γ -carboxyl radical of the glutamyl unit, which is required for the activity of pepsin, is neutralized by the free α -amino-group of the same amino-acid unit. These results are summarized in Table 4.

Trypsin can act at peptide linkages adjacent to either an arginine or a lysine unit and replacement of these basic amino-acid residues by others yields resistant products. The second amino-group of the dibasic amino-acid unit must be unsubsti-

tuted, for trypsin acts upon α -benzoyl-L-arginine :amide and α -benzoylglycyl-L-lysine :amide, for example, but not upon α -benzoylglycyl-(ϵ -carbobenzoxy-)-L-lysine amide, in which both the amino-groups of the lysine unit are covered.

TABLE 4. ACTION OF PEPSIN AND CHYMOTRYPSIN
UPON SYNTHETIC PEPTIDES

Substrate	Action of	
	Pepsin	Chymo- trypsin
Carbobenzoxy-L-glutamyl-:L-tyrosyl-:glycine amide	+	+
Carbobenzoxy-L-tyrosyl-:glycine amide	-	+
Carbobenzoxy-L-phenylalanyl-:glycine amide	-	+
Carbobenzoxy-L-glutamyl-:L-tyrosyl-glycine	+	-
L-Glutamyl-L-tyrosyl-:glycine amide	-	+

To sum up, we may say that *pepsin* can act at peptide links formed between the α -carboxyl group of a dicarboxylic amino-acid and the α -amino-radical of an aromatic amino-acid, but requires that the second acidic group of the dicarboxylic acid shall be free, and is inhibited if there is an amino-group nearby. *Chymotrypsin* can act upon peptide bonds formed from the α -carboxyl group of an aromatic amino-acid, but is inhibited if there is a carboxyl radical in the immediate vicinity, while *trypsin* can act upon peptide links formed from the carboxyl group either of arginine or of lysine, but requires that the second amino-group of the dibasic amino-acid unit shall be free. This enzyme appears to be inhibited by nearby α -amino or carboxyl radicals.

It must not be supposed, however, that these enzymes cannot attack peptide linkages other than those determined by Bergmann, for Harington has already shown that bonds of other types can be attacked by pepsin. In Harington's work it was found that an aromatic amino-acid is required, but that the free carboxyl grouping of glutamic acid can be replaced by the sulphhydryl of cysteine. Both tyrosyl-cysteine and cysteinyl-tyrosine, for example, were attacked by pepsin, though more slowly than the corresponding *N*-carbobenzoxy derivatives. Even so, the fact remains that pepsin cannot act upon any arbitrary peptide linkage, but is restricted, probably, to linkages of only a few special types. Clearly, therefore, the endopeptidases

are very exacting indeed and, contrary to earlier opinion, able to act only upon bonds of certain types. The specificity requirements established by Bergmann are summarized in Fig. 10.

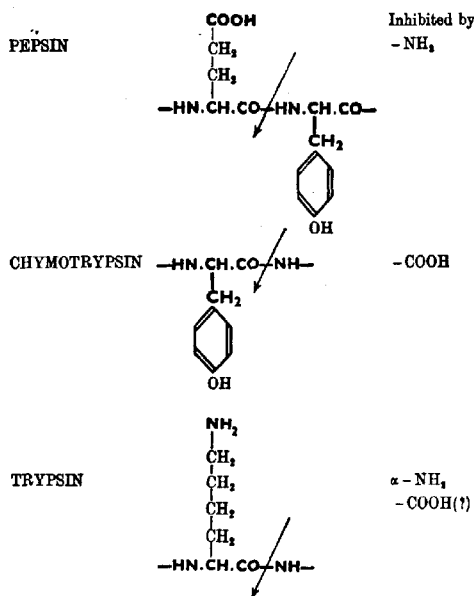
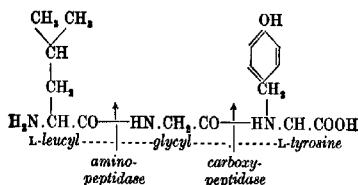


Fig. 10. Specificity requirements of endopeptidases. The essential requirements are printed in heavy type: the point of attack is indicated by an arrow in each case.

By their concerted action the endopeptidases divide intact protein molecules into smaller fragments, and the stage is set for the action of two members of the group of exopeptidases, viz. carboxypeptidase and aminopeptidase. These two enzymes catalyse the splitting only of terminal peptide bonds, with consequent liberation of the terminal amino-acid units. *Carboxypeptidase* removes the terminal unit of which the carboxyl radical is free, *aminopeptidase* acting at the other end of the chain, where the

terminal unit has a free amino-group. Thus in L-leucyl-glycyl-L-tyrosine, for example, we have the following structural arrangement:



Carboxypeptidase acts upon this tripeptide to liberate tyrosine, and aminopeptidase to produce free leucine. Carboxypeptidase requires a free carboxyl radical for its action to take effect, aminopeptidase requiring a free amino-group. Carboxypeptidase is unable to act if there is a free amino-group nearby, while aminopeptidase is similarly affected by a free carboxyl radical. Neither enzyme, therefore, attacks the dipeptide left after the other has attacked the original tripeptide, nor will either enzyme attack peptides containing amino-acid residues of the D-series.

In these two peptidases, then, we can recognize specificity requirements which include the presence of the right terminal radical, which must be unsubstituted, and the absence from the immediate vicinity of electrically opposite radicals, together with the usual stereochemical requirements. It is believed, however, that the requirements of these peptidases are more exacting even than this, and there exist more than one amino- and more than one carboxypeptidase, each with special requirements.

Whereas the endopeptidases split large protein units into smaller fragments and produce few free amino-acid molecules in the process, the amino- and carboxypeptidases liberate free terminal amino-acid units one by one until only dipeptides remain. These, as we have seen, are not further attacked by these enzymes, but are split in their turn by *dipeptidase*. Probably there are several dipeptidases, each specific for certain individual dipeptides or groups of dipeptides. Like all the other digestive peptidases, dipeptidase requires that the constituent amino-acids of its substrates shall be members of the L-series, so that, of the

four possible alanyl-leucines, the L-L-, L-D-, D-D- and D-L-, only one is attacked, namely, L-alanyl-L-leucine.

In passing the reader may be reminded that peptidases capable of attacking peptides containing amino-acids of the D-series are now known to exist, but the foregoing description of the digestive peptidases holds, even though it cannot be extended to cover all kinds of peptidases.

Rennin. In the gastric juice of young mammals we find another proteolytic enzyme, rennin. Like pepsin, this enzyme is secreted in the form of an inactive precursor, pro-rennin, which is activated by hydrochloric acid. The optimum pH for activation is considerably less acid than that for pepsin. The most characteristic feature of rennin is its milk-clotting power, and it is, in fact, the active principle of commercial preparations of 'rennet'. It catalyses the conversion of the milk casein ('caseinogen') into another product, paracasein ('casein'), the calcium salt of which is insoluble so that, in the presence of the calcium of the milk, a firm clot or curd is formed. Rennin from the abomasum (fourth stomach) of the calf has recently been obtained in crystalline form, but little has as yet been discovered about its specificity requirements. Like pepsin, rennin has proteolytic properties, but has a more alkaline pH optimum: its optimal pH when acting upon haemoglobin, for example, is 3.7 as against about 2.0 for pepsin.

Intracellular Peptidases

The presence of intracellular enzymes capable of catalysing the hydrolysis of peptides of greater or less chemical complexity and molecular weight has been demonstrated in many animals and plants. In many cases these enzymes are believed to have digestive functions, especially among the lower animals, many of which produce no digestive secretions but take particulate food by phagocytosis and digest it intracellularly. There is a considerable literature on this subject, and it may be said that enzymes resembling pepsin and trypsin, at least in their general properties, are present in the cells, but not much is known about them.

Other intracellular proteolytic enzymes include such plant enzymes as papain, ficin and bromelain, obtained from the sap or latex of the paw-paw, fig and pineapple respectively. Probably all of these are complex mixtures. Much more is known about the intracellular, autolytic enzymes of animal tissues, known collectively as kathepsin. Kidney and spleen tissues offer good sources of kathepsin, but it is present in many other organs and also in tumours of various kinds. Most of our recent knowledge of the kathepsin group we owe to Bergmann and his collaborators, who have shown that kidney and spleen kathepsins comprise at least four components, the specificities of each of which have now been studied. Of these, kathepsin I is homospecific with pepsin, i.e. has the same specificity requirements as pepsin; kathepsin II is homospecific with trypsin, while components III and IV are homospecific with aminopeptidase and carboxypeptidase respectively.

Although a quantitative as well as a qualitative homospecificity has been demonstrated between the digestive peptidases and the components of kathepsin, the enzymes are probably not identical. The kathepsins are not activated by unmasking, as in the case of the digestive endopeptidases, nor yet by heavy metals such as manganese, as in the case of dipeptidase. On the contrary, the intracellular peptidases are inactivated by heavy metals, but can be activated by the addition of cyanide, hydrogen sulphide, cysteine, glutathione and sometimes by ascorbic acid. It is now widely believed that the active form of papain, for example, is a complex formed between the enzyme-protein and an —SH compound, probably papain-cysteine.

It has already been suggested that these intracellular peptidases most probably function to maintain a dynamic equilibrium between the cell proteins and simpler products present in the cell contents. We have seen that the extracellular, digestive peptidases constitute a set of tools whereby the proteins of the food can be completely dismantled, and if it could be shown that the action of enzymes of this kind is reversible, we should feel more confident that the kathepsins, with which they are homospecific, constitute an outfit capable of reconstituting as well as

degrading proteins. Bergmann has succeeded in demonstrating synthetic activity on the part of several enzymes and has shown, for example, that chymotrypsin catalyses the condensation of benzoyl-L-tyrosine with glycylalanide to yield benzoyl-L-tyrosyl-glycylalanide. The product in this case is insoluble and is precipitated, so that the hydrolytic action of the enzyme does not seriously oppose its synthetic performance. Other syntheses have been accomplished with, for example, papain-cysteine, i.e. papain activated by the addition of cysteine, and an example can be seen in Fig. 3B (p. 23).

CARBOHYDRASES

Enzymes capable of catalysing the breakdown of carbohydrates are very widely distributed indeed, and occur both in digestive secretions and within the cells of animals, plants, and micro-organisms of many and perhaps all kinds. They may be considered under two main headings, the *polysaccharases*, which act upon the large molecules of polysaccharides such as starch and glycogen, and the *glycosidases*, the substrates of which are small molecules such as various di- and trisaccharides, in addition to glycosides of other kinds.

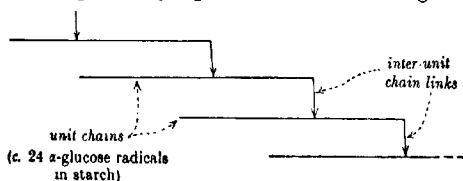
Amylases

Polysaccharases

Most is known about the amylases, which act upon starch and glycogen but not upon cellulose. Plant amylases such as that of malt have been resolved into two components known respectively as α - and β -amylases. In the ordinary course of events these enzymes act together to catalyse a quantitative conversion of starch or glycogen into the disaccharide, maltose. They act, however, in quite different ways, and in order to appreciate the differences between them it is necessary to have in mind a fairly clear picture of the probable structure of starch and the related polysaccharide, glycogen.

Starch is considered to be a mixture of two main components, amylose and amylopectin. Amylose, which usually accounts for only a small part of whole starch, has a long, coiled molecule consisting of 200–300 α -linked glucose radicals. In amylopectin,

on the other hand, we find chains containing c. 20 or 24 glucose radicals, linked again in the α -manner. An unknown number of these 'unit chains' are joined together to make amylopectin. We cannot say at the present time exactly how the 'inter-unit chain' linkages are arranged, except that the terminal radical of each unit chain appears to be united through its reducing hydroxyl group to some hydroxyl group of an adjacent unit chain, located in part at least in position 6. The structure of amylopectin can be pictorially represented in the following manner:



Glycogen appears to contain nothing analogous to amylose, but consists of a large number of unit chains, each containing 12, or sometimes 18, α -glucose units, and these unit chains are believed to be linked together as in amylopectin.

In considering the modes of action of the α - and β -amylases it should be clearly realized that the use of the prefixes α - and β - is not meant to imply that these enzymes act upon α - and β -glucosidic links respectively. β -Amylase, more appropriately termed the *maltogenic amylase*, acts upon the long, open chain of amylose to remove pairs of glucose radicals, so that the whole is converted into maltose. When presented with amylopectin, however, its action begins at the free ends of the unit chains, but ceases when the inter-unit chain linkages are approached. In this case, about 50% of the amylopectin is converted into maltose. The residual product, the so-called α -amylodextrin, gives a 'port wine' colour with iodine, unlike whole starch. This dextrin, which is resistant to the maltogenic amylase, can be attacked, however, by the *dextrinogenic*, or α -amylase, which splits it into smaller fragments, which are still of the dextrin type but no longer give any coloration with iodine. α -Amylase received its alternative title of dextrinogenic amylase because,

acting upon amylopectin, it yields only comparatively small dextrin-like molecules in the first instance. If it is allowed to act for long periods, about 85 % of starch is eventually split into maltose, though the rate of this secondary process is so small that it may perhaps be due to incomplete removal of maltogenic amylase from the preparations. Neither component alone can convert starch or glycogen completely into maltose: this is only accomplished when both amylases act together. The action of the dextrinogenic amylase may include some splitting of the inter-unit chain linkages, since the dextrans produced when it acts upon starch or α -amylodextrin can then be further attacked by the maltogenic enzyme and converted completely into maltose.

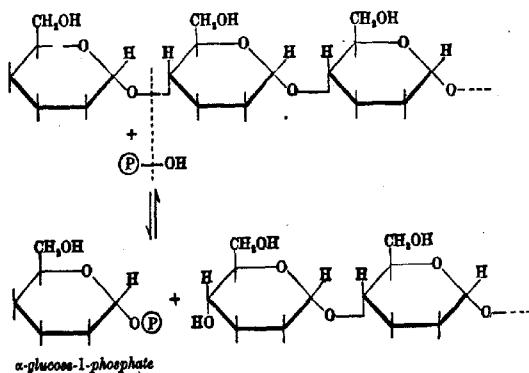
The digestive (salivary and pancreatic) amylases of animals have not yet been resolved into dextrinogenic and maltogenic components, though they can carry out a considerable conversion of starch into maltose. They behave like mixtures of dextrinogenic and maltogenic amylases, with the dextrinogenic enzyme preponderating. In malt, however, the maltogenic enzyme is the more abundant. It has long been known that salivary and pancreatic amylases lose their activity if dialysed, and the case is often quoted in illustration of the importance of inorganic ions in the activation of certain enzymes. This, however, is not entirely a true bill, for while it is true enough that dialysed salivary amylase, for example, is no longer active under conditions which were formerly optimal, it is still weakly active at more acid pH values. There is, it is also true, a considerable loss of total activity, but the removal of chloride ions does not deprive the enzyme of its power to activate the substrate, but appears to alter its physical state in some way. In all probability the enzyme dissociates in a different manner in the absence of chloride ions (see p. 51) and, if this is so, it might perhaps be expected that the replacement of chloride by other ions would lead to a displacement of the optimum pH. This does in fact happen, as is shown by the curves of Fig. 9 (p. 45).

Phosphorylases see page 57.

Many animal and plant tissues, notably mammalian liver, contain autolytic enzymes which lead to the post-mortem con-

version of glycogen into glucose. This suggests at first sight that there must exist intracellular amylases which can catalyse the synthesis of glycogen under normal intracellular conditions, but no evidence has so far been adduced to show that the maltogenic and dextrinogenic amylases just discussed can act reversibly. Nevertheless, it is certain that plant and animal tissues can produce starch or glycogen respectively from glucose, and for some years it was usually supposed that the synthesis must involve enzymes similar to those known to be concerned in the digestive hydrolysis of these polysaccharides.

A series of investigations, carried out by Hanes in England and by Cori and Cori in America, has shown that the intracellular synthesis and breakdown of starch and glycogen follow paths that are quite different from those of digestive hydrolysis. The degradation is not, in fact, a process of hydrolysis but one of phosphorolysis, in which the glucosidic bonds are not broken by the elements of water ($\text{HO}-\text{H}$) but by those of phosphoric acid ($\text{HO}-\text{P}$), while the product of breakdown is not maltose nor even glucose, but α -glucose-1-phosphate. Hanes and the Coris succeeded in obtaining preparations containing the phosphorylases of peas, potatoes, liver, muscle and other tissues, and were able to show that they catalyse the decomposition of starch and glycogen according to the following equation:



If α -glucose-1-phosphate is added to a suitable preparation of a phosphorylase, polysaccharides can be synthesized, but the enzyme does not act upon β -glucose-1-phosphate. Phosphorylases from peas and potatoes yield granular products closely resembling natural starch, while the liver enzyme yields a polysaccharide very similar to liver glycogen. Muscle phosphorylase, however, forms a product which superficially resembles starch rather than muscle glycogen in that it gives a blue instead of a red coloration with iodine, and is precipitated in granular form as the synthesis proceeds. An interesting feature of these syntheses is that the phosphorylases cannot act upon α -glucose-1-phosphate except in the presence of a little added starch or glycogen: presumably, therefore, the enzyme needs a suitable 'anchor' to which the new glucose units can be attached.

In the ordinary way, glycogen is stored in the livers of animals and distributed, under very precisely defined conditions, to other tissues by way of the blood, travelling in the form of free glucose. In all probability this glucose arises in the liver by the dephosphorylation of α -glucose-1-phosphate, which itself arises by the phosphorolysis of liver glycogen. In the muscles, where blood glucose is used as source of newly formed muscle glycogen, it is probable that the blood glucose must first of all be converted back into α -glucose-1-phosphate, which is then built up again into glycogen. This seems at first sight a rather roundabout way of transferring glycogen from one place to another, but we shall see later on that there seem to be good reasons why the task is accomplished in this, rather than in some other manner.

One lesson of considerable importance is inherent in all this. Although the enzymes that catalyse the digestive hydrolysis of starch and glycogen must, on theoretical grounds, be regarded as capable of working reversibly, it has not so far proved possible to synthesize polysaccharides with their aid. This must presumably mean that the energy conditions are all in favour of breakdown and against synthesis, and that we have not as yet discovered a way in which energy can be pumped into the system in such a manner as to force the reaction to proceed synthetically. Since starch and glycogen are freely interconvertible with

α -glucose-1-phosphate, it seems that the phosphorylated glucose must have a higher initial energy-potential than the free sugar and can serve as starting material for the synthesis for that reason. As we shall see, there is evidence that energy has to be expended in the conversion of free glucose into its phosphorylated forms.

Cellulase

Although cellulose forms a very large part of the food of herbivorous animals, remarkably few animals of any kind possess any enzyme or enzymes capable of catalysing its hydrolysis. Cellulose-splitting enzymes have been described in the digestive secretions of a number of herbivorous gastropods, including terrestrial forms like *Helix* and aquatic species such as *Strombus*, *Pterocera* and *Aplysia*, and cellulases appear to be present also in the digestive juices of a few wood-eating insects. The ship-worm, *Teredo*, is an interesting creature from this point of view, for its digestive gland contains cells which appear to be specialized for the phagocytic ingestion and intracellular digestion of fine particles of wood which the animal scrapes off as it bores. But in the vast majority of cases it is nevertheless true that animals do not produce cellulase, even when they depend largely upon cellulose as a primary source of food.

This paradox is due to the fact that most cellulose-eating animals do not digest cellulose for themselves, but maintain in their alimentary tracts large populations of symbiotic micro-organisms, including bacteria, protozoa and yeasts, which play a very important part in their nutrition. Many of these micro-organisms are capable of degrading cellulose, and recent work on the processes of digestion in ruminants has shown that cellulose is broken down with production of large amounts of lower fatty acids by the symbionts. Acetic and propionic acids predominate, and are accompanied by formic, butyric and valeric acids, together with large volumes of carbon dioxide, methane and hydrogen. The ruminant thus obtains fatty acids rather than sugars from the cellulose it consumes, and has to repay the micro-organisms which carry out the conversion by providing them with *Lebensraum* in the form of a capacious caecum, a

multiple stomach, or some other commodious dilatation of the alimentary canal. Similar processes are believed to take place in many wood-eating insects and other herbivores.

Little is known about the enzymes whereby these symbiotic organisms break down the cellulose. Presumably they must include a cellulase, and it has indeed been shown that the free-living protozoan, *Vampyrella*, secretes an extracellular cellulase which attacks the cellulose of the cell walls of the *Spirogyra* which furnishes its food. The secretion of extracellular cellulases must probably play an important part in the early stages of microbial attack upon cellulose, which is so insoluble as to require some extracellular comminution before it can be got into the cells for further chemical manipulation. Cellulose-splitting enzymes, and enzymes capable of synthesizing cellulose certainly occur in plants and fungi, and must be of very great importance in plant economy, but we have very little information about them.

It is said that preparations containing cellulase will also act upon the animal polysaccharide, chitin, in which the β -linked glucose radicals of cellulose are replaced by similarly linked units of *N*-acetyl glucosamine. Whether or not cellulase and chitinase are identical has not been determined.

Other polysaccharases include enzymes capable of splitting polyfructofuranosides such as inulin and levan, and enzymes that act upon polysaccharides such as the mannans, pectins and so on, but we have only very scanty information about these.

Glycosidases

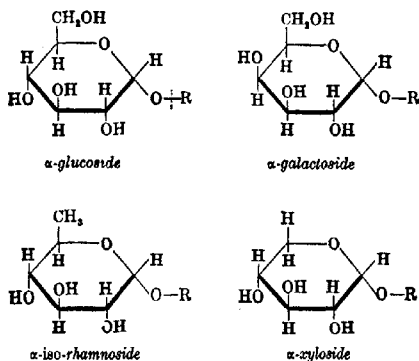
We know of many enzymes capable of splitting simple glycosides, all of which show a high order of specificity towards the glycosidic part of the substrate molecule. Of these the most important are the glucosidases and the saccharases.

α -Glucosidases

α -Glucosidases of two types are known. The most specific of these are the 'true' maltases, which act only upon a single α -glucoside, viz. maltose (glucose-4- β - α -glucoside). Enzymes of this kind occur in malt and in *Aspergillus*. The more widely distributed digestive 'maltases' of animals, and the 'maltase'

of yeast, are able to act upon α -glucosides other than maltose and should therefore be called α -glucosidases rather than maltases. These enzymes will act upon such substances as α -methyl- β -glucoside and sucrose (α -glucosido- β -fructofuranoside), though they are without action upon the α -glucosidic linkages of the large molecules of starch and glycogen.

The order of specificity of these α -glucosidases is very high, for a completely unmodified α -glucosido-radical is required in their substrates: β -glucosides, α -galactosides, α -xylosides and α -iso-rhamnosides are not attacked, in spite of their close structural resemblances to the α -glucosides:



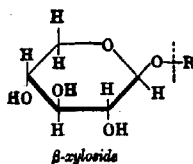
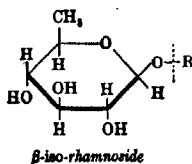
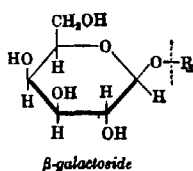
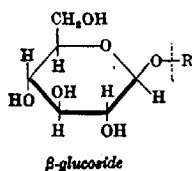
β -Glucosidases

Many cells and tissues have been shown to contain β -glucosidases. The classical source of such an enzyme is the 'emulsin' of bitter almonds. The latter contain amygdalin, a β -glucoside which, when attacked by the β -glucosidase component of emulsin, yields mandelonitrile, which is then attacked by another enzyme, mandelonitrilase, to give free hydrocyanic acid. Sweet almonds also contain these enzymes, but no amygdalin is present in this case.

β -Glucosides are as common in nature, especially in plant materials, as their α -counterparts are rare, and among the more

interesting of these we may mention salicin (salicyl- β -glucoside) and a β -glucoside of indoxyl which occurs in the indigo plant and gives rise, after hydrolysis and oxidation, to natural indigo. There are many others. In addition, a number of the simpler saccharides are β -glucosides, notably cellobiose (glucose-4- β -glucoside), which stands in the same relation to cellulose as maltose does to starch and glycogen, and gentiobiose (glucose-6- β -glucoside), which occurs naturally in combination with mandelonitrile in the form of amygdalin.

β -Glucosidases obtained from different plant and animal sources have much in common. In particular, it may be pointed out that their specificity, though high, is less marked than that of the α -glucosidases. Although 'cellobiases' have been described from time to time there is no reason to think that they are comparable with the 'true' maltases of malt and *Aspergillus*, but rather that they are group-specific β -glucosidases. These enzymes are rather less specific than the corresponding α -glucosidases, which require a completely unmodified α -glucosidic radical in their substrates. The specificity requirements of the β -glucosidases do not extend as far as carbon 4, for some β -galactosides are also split. Modifications can also be made at position 6, for β -iso-rhamnosides and β -xylosides are also split, though less rapidly than the normal substrates:



Saccharases are very widely distributed indeed in the digestive secretions of animals, in plants and in many micro-organisms, though not, apparently, in the cell contents of animals. Two types can be distinguished, the *glucosaccharases* (in digestive secretions of animals and in *Aspergillus*) and the *fructosaccharases* (in yeast).

Sucrose itself is α -glucosido- β -fructofuranoside, and the molecule may be attacked from either end. The glucosaccharases are competitively inhibited by glucose, which may therefore be presumed to compete with sucrose for possession of the combining groups of the saccharase molecule. The glucosaccharases are, in fact, α -glucosidases. Similarly, the fructosaccharases are competitively inhibited by fructose, and may therefore be supposed to act from the fructose end of the sucrose molecule. Highly purified yeast saccharase is able to attack methyl- β -fructofuranoside and is thus a β -fructofuranosidase.

The differences in specificity between these types of saccharases are shown even more clearly by the fact that fructosaccharase attacks the trisaccharide raffinose (β -fructofuranosido- α -glucosido-6- α -galactoside) while glucosaccharases do not. Another trisaccharide, melezitose (melicitose: α -glucosido- β -fructofuranosido-6- α -glucoside) is split by glucosaccharase and not by fructosaccharase.

Other glycosidases are known. They include α -galactosidases, which attack compounds such as raffinose (β -fructofuranosido- α -glucosido-6- α -galactoside), and β -galactosidases which act upon compounds such as lactose (glucose-4- β -galactoside). Brewers' yeast, for example, contains an α -galactosidase ('melli-biase'), while bakers' yeast, *Aspergillus* and the digestive secretions of animals contain β -galactosidases ('lactases'). Mannosidases also are known.

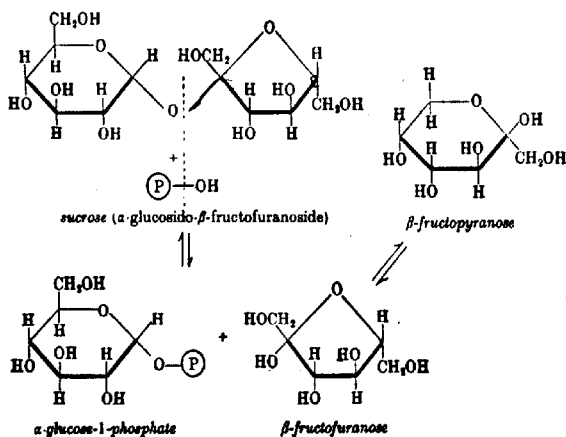
Reversibility of the glycosidases

A number of β -glucosides have been synthesized enzymatically with the aid of emulsin. In such cases, e.g. in the synthesis of gentiobiose, strong solutions of the reactants are taken in order to force the reaction backwards in accordance with the principle

of the mass law. Claims have also been made for the synthesis of sucrose from strong solutions of glucose and fructose, but these seem to be less well supported by the experimental evidence. Whether or not they are true can only finally be decided when sucrose is actually isolated from the reaction mixtures.

Whereas the amounts of sugars such as gentiobiose are fairly large under equilibrium conditions, the amounts of sucrose present at equilibrium are certainly small, and it must be supposed that the free-energy conditions favour the hydrolysis rather than the synthesis of sucrose, just as seems to be the case in the breakdown and synthesis of starch from glucose. Sucrose is, nevertheless, synthesized on a large scale by many plants, and as yet we have very little knowledge about the mechanisms involved. It does seem improbable that the process can be catalysed by saccharase with glucose and fructose as starting materials.

It has, however, been shown that certain bacteria can catalyse a synthetic production of sucrose from α -glucose-1-phosphate and free fructose. The process, which is freely reversible, is one of phosphorolysis and may be compared with the phosphorolysis of starch and glycogen (p. 73):



Here, as in the synthesis of starch and glycogen, the starting materials have a higher free energy, or 'energy potential', than the free sugars. Free fructose exists mainly in the stable pyranose form, and energy is presumably needed to force the 6-membered pyranose ring to take up the 5-membered furanose configuration.

LIPASES AND ESTERASES

Under this heading we have to consider several distinct groups of enzymes. There are, first of all, the very widely distributed *lipases* and *esterases* which catalyse the hydrolysis of esters of alcohols with organic acids and, in addition, several groups which catalyse the hydrolysis of esters formed between alcohols and inorganic acids. Of these the *phosphatases* and *sulphatases* may be mentioned, phosphatases of various kinds being almost universally distributed and of enormous metabolic importance.

There are sharp differences of specificity with respect to the acid component of the ester. Phosphatases are group-specific as a rule, and act only upon organic esters of phosphoric acid, such, for example, as phosphoglycerol and hexosemonophosphate, and are without action upon organic sulphates or esters of organic acids. Lipases, on the other hand, are enzymes of low specificity. They act only upon esters of organic acids, such as ethyl acetate and the fats, but provided the acid is organic in nature its precise chemical identity, and that of the alcoholic radical, are matters of relative indifference.

Lipases

Lipases occur in the gastric, pancreatic and intestinal juices of vertebrates, and have been reported also in the digestive secretions of many invertebrates. They also occur in plants, especially in seeds, and have been found in other organisms such as *Aspergillus* as well. Their specificity is very low, and any lipase will split virtually any wholly organic ester. The acid component may be anything from acetic to palmitic, stearic, or even higher fatty acids, and may be saturated or unsaturated. Similarly, the alcohol may be a short-chain compound such as methyl or ethyl alcohol, or a long chain such as cetyl alcohol (C_{16}).

It may be monohydric or, as in the case of glycerol, polyhydric, and esters even of complex alcohols such as cholesterol are also attacked. So low is the specificity of these enzymes that many attempts have been made to fractionate them, but without success. In many other cases, notably that of 'erepsin', it has been found that enzymes of seemingly low specificity consist, in reality, of mixtures of enzymes, each individual catalyst being more specific than the mixture, but this has not so far been found to be true of the lipases.

Lipases from different sources split different esters at different relative rates. Apparently the liver enzyme prefers esters of short-chain fatty acids, while the pancreatic enzyme prefers esters of long-chain acids. Gastric lipase and the lipase of the castor-oil bean (*Ricinus* lipase) resemble the pancreatic enzyme in this respect. Enzymes of the type found in liver are sometimes called *esterases* to indicate their apparent preference for simple esters as opposed to fats.

Although their specificity towards the structure of the substrate is very low, the lipases show marked stereochemical specificity when allowed to act upon esters containing an asymmetric carbon atom, such, for example, as the ethyl esters of the mandelic acids, $C_6H_5CH(OH)COOH$. One isomer is always more rapidly attacked than the other in cases of this kind, whether the asymmetric carbon atom is situated in the alcoholic or in the acidic component of the ester.

Some authors have suggested that bile salts, which play an important part in the digestion and absorption of fatty substances, may act as activators for the digestive lipases, but this is open to doubt.

An important feature of the lipases is the readiness with which they will catalyse the synthesis as well as the hydrolysis of fats and esters. If a fat such as triolein is incubated under suitable conditions with, say, pancreatic lipase, the reaction does not go to completion. Instead, an equilibrium is approached, and the composition of the final reaction mixture is the same from whichever side it is approached. Fig. 11 shows the results of experiments on the action of *Ricinus* lipase on triolein, in one case,

and upon a mixture of glycerol and oleic acid in the other. Much of what we know about the reversibility of enzymic reactions was first learned from studies of systems of this kind. That pancreatic and intestinal lipases do not carry the digestion of fats to completion under *in vitro* conditions does not mean, of course, that much of the food fat fails to be digested under the conditions obtaining *in vivo*, for the products of digestive hydrolysis are removed by absorption as fast as they are formed, so that the forward reaction is not opposed, as it would be if the products were allowed to accumulate.

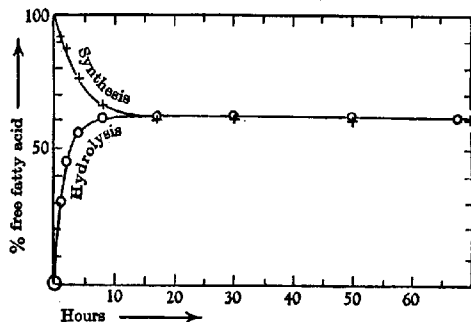


Fig. 11. Hydrolysis and synthesis of triolein by *Ricinus* lipase.
(After Parsons; data of Armstrong and Gooney.)

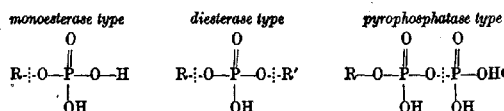
The three fatty acid radicals of a typical triglyceride are not removed simultaneously, but come off one after another under the influence of the digestive lipases, so that di- and monoglycerides are produced as intermediate products and this, as we shall see, is very important in connexion with the absorption of fatty food materials from the small intestine in animals.

Mention must also be made of a curious enzyme found in the venoms of the cobra and the rattlesnake. This acts upon lecithins, in which, it will be recalled, two of the alcoholic groups of glycerol are esterified by fatty acids and the third by phosphocholine. The ordinary digestive lipases are able to remove both of the fatty acid radicals, but the so-called *lecithinases* of these snake venoms remove one only, and yield a product in which the

remaining fatty acid is of the saturated type. This compound is called lysolecithin, on account of its powerful haemolytic action. The undesirable consequences of being bitten by these reptiles include a large-scale lysis of the red blood cells and consequent disturbances of the respiratory functions of the blood. The venom of vipers, by contrast, causes intravenous clotting of the blood, a process which is catalysed by a proteolytic rather than a lipolytic enzyme.

Other esterases of more specific nature include *choline esterase*, an enzyme of great importance in animal tissues, in which it serves to catalyse the hydrolysis of acetylcholine, the neuro-hormone of the parasympathetic nervous system.

Phosphatases appear to be almost universally distributed, but relatively little is known about them. There appear to be three distinct types, which may be termed phosphomonoesterases, phosphodiesterases and pyrophosphatases, catalysing the hydrolysis of substances of the following general types:



(R and R' represent alcoholic radicals). A very important example of the pyrophosphatase type is the *adenosine triphosphatase* of muscle. This enzyme is closely associated with *myosin*, the contractile protein which makes up the bulk of the muscle substance. Bakers' yeast contains an interesting pyrophosphatase which appears to be wholly specific for the hydrolysis of inorganic pyrophosphates and is devoid of action upon adenosine triphosphate and other organic pyrophosphates.

Generally speaking, phosphatases of the monoesterase type seem not to be specific with respect to the nature of the alcoholic radical, and act alike on many organic phosphates such, for instance, as phospho- β -glycerol, α -glucose-1- β -phosphate and glucose-6- β -monophosphate. The reactions they catalyse may be generally expressed as follows:



One such enzyme plays an important part in the ossification of cartilaginous structures such as the bones and teeth. Unossified cartilage and cartilaginous structures which do not undergo ossification contain no phosphatase, but the enzyme makes its appearance just at the time that ossification sets in. Acting upon organic phosphates present in the blood, the enzyme is thought to catalyse a localized liberation of phosphate ions, which unite with calcium ions, also provided by the blood, to form the insoluble calcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$. This is deposited in an ordered, crystalline manner in the cartilaginous matrix. The more rapidly ossification proceeds the higher is the phosphatase activity of the tissue. An interesting point in this connexion is that the calcified structures of the cartilaginous fishes (*Elasmobranchii*) contain an active phosphatase, just as do those of the bony fishes (*Teleostei*). The differences between the mechanical properties of calcified cartilage and true bone appear to be due to differences in the manner in which the calcium phosphate is deposited in the two cases.

In addition to being present in cells of nearly every kind, phosphatase is present in milk. If milk is pasteurized in the correct manner, the treatment accorded is just sufficient to inactivate the milk phosphatase, so that the absence of phosphatase activity may be taken as an indication that the process has been properly carried out. The method used for routine testing depends upon the hydrolysis of phenyl- β -phosphate with formation of free phenol.

The group of phosphatases includes some of the enzymes concerned in the hydrolysis of nucleic acid. *Nuclease*, recently crystallized, appears to be a kind of pyrophosphatase, but its specificity has not yet been thoroughly examined. This enzyme splits nucleic acids into their component nucleotides. The so-called *nucleotidases* appear to be phosphatases of the monoesterase type, and these split off phosphate to liberate the corresponding nucleosides.

Among the phosphatases are many enzymes that require magnesium ions as activators. Very minute concentrations of Mg^{++} are all that are required in many cases, and the mode of action of these ions is still obscure.

OTHER HYDROLYTIC ENZYMES

Arginase, a very important enzyme of which larger or smaller concentrations are present in most animal cells, catalyses the hydrolytic deamidation of arginine to yield urea and ornithine (see p. 13), and is believed to play a central part in the mechanisms whereby urea is synthesized in the mammals. It is thought to be a manganese-containing protein, and is one of the most specific hydrolases known. Arginase is very powerfully inhibited by ornithine, though not by urea, a circumstance which suggests that it must combine with the ornithine radical. Its optimum pH lies far in the alkaline range, probably about pH 10, at which it is very unstable.

Urease occurs in large concentrations in certain seeds, notably in jack- and soya-beans, from which it is usually prepared. It has been found in numerous other plant tissues and in the tissues of a few invertebrates, though it appears to be totally lacking from vertebrate organisms. Urease had the distinction of being the first enzyme to be obtained in the crystalline state. It was extracted from jack-bean meal by Sumner, purified and crystallized in 1926, and shown to be a protein. Crystalline urease gives all the usual tests for proteins apart from the Mølich reaction, so that it may be presumed to contain no carbohydrate groupings.

Urease possesses a number of very unusual properties. Unlike most enzymes, it is inhibited by high concentrations of its substrate, an effect which can be abolished by the addition of glycine, as is shown in Fig. 12. This phenomenon is usually explained on the supposition that, in addition to combining with urea to form a reactive complex, ES , which is then hydrolysed, it tends to combine with a second molecule of urea when the concentration of the latter is high to form a stable complex, ES_2 . Another, and possibly a unique feature is that the optimum pH of urease is not fixed but proportional to the logarithm of the substrate concentration.

The specificity of urease is very high. Its action has been tested upon a large number of substituted ureas, none of which

appears to be attacked, with possible though dubious exceptions in the cases of the *sym.* dimethyl- and diethyl-ureas.

Hydrolytic deaminases and deamidases other than urease are also known. *Adenase*, which catalyses the hydrolytic deamination of adenine to yield hypoxanthine, and *guanase*, which converts guanine into xanthine in like manner, are present in the liver tissue of mammals, and must most probably occur elsewhere.

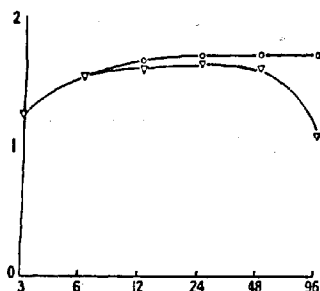
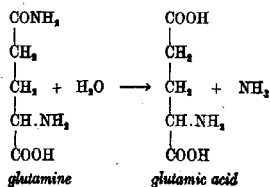


Fig. 12. Influence of urea concentration on activity of urease (v). The inhibitory influence of high concentrations of urea is counteracted by 0.2% glycine (o). Ordinate: initial velocity of hydrolysis. Abscissa: concentration of urea. (After Haldane, from Kato, 1923.)

Another important enzyme concerned with the metabolism of purine derivatives is the *adenylic deaminase* of muscle. This enzyme, which is not identical with adenase, catalyses the hydrolytic deamination of adenylic acid to yield inosinic acid.

Animal tissues contain a powerful *glutaminase* and plants an homologous *asparaginase*. These enzymes catalyse the hydrolysis of the amides of the dicarboxylic amino-acids, e.g.

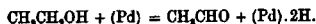


CHAPTER IV

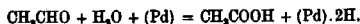
OXIDIZING ENZYMES

THE OXIDATION OF ORGANIC COMPOUNDS

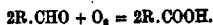
CHEMICAL compounds may be oxidized in a number of different ways. Oxygen may be added to the molecule, as when hydrogen is oxidized to form water, or hydrogen may be removed, as when hydrogen sulphide is oxidized to free, elemental sulphur. Again, oxidation can be effected by the removal of electrons, as when ferrous salts containing Fe^{++} are oxidized to the corresponding ferric compounds, in which Fe^{+++} is present. Among organic compounds, however, oxidation most commonly takes place by the removal of hydrogen, i.e. by the process known as *dehydrogenation*. This conclusion was first reached as the result of an extensive series of studies by Wieland, who drew attention to the fact that if colloidal palladium is added to aqueous solutions of many organic compounds, catalytic oxidation ensues, the palladium becoming charged with hydrogen in the process. In the case of ethyl alcohol, for example, the reaction proceeds in the following manner:



Aldehydes can also be oxidized in this manner, but in this case a molecule of water is involved:

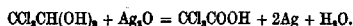


The oxidation of aldehydes is a matter of great biochemical importance, as we shall see, and it might have been anticipated that their oxidation would proceed by the addition of oxygen directly to the aldehyde molecule as follows:

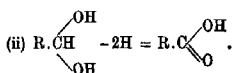
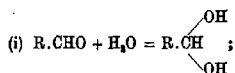


Wieland tested this possibility by treating chloral dissolved in benzene with dry silver oxide, and found that no oxidation takes

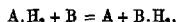
place. If, however, chloral hydrate was substituted for chloral itself, it was oxidized according to the following equation:



Evidently, therefore, the water molecule plays an integral part in the process, and it is now generally believed that in aqueous solution the aldehydic radical can become associated with a molecule of water, though it is only rarely, as in the cases of chloral and glyoxylic acid, that the aldehyde hydrate is sufficiently stable to be isolated. The following general equations can therefore be written to describe the oxidation of aldehydes:



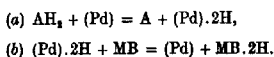
It must be remembered that whenever one substance is oxidized at the expense of another, the oxidation of the first is necessarily attended by the reduction of the second. Hence it is usual to speak of an 'oxidation-reduction reaction', sometimes abbreviated 'O/R reaction'. If the process is a biological one it can usually be represented in general terms by the equation:



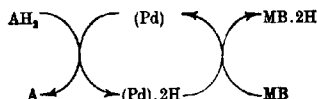
and we can define a number of terms which are in general use in discussions of reactions of this kind. The reductant, AH_2 , is known as the *hydrogen donator*, and the oxidant, B, as the *hydrogen acceptor*. A third factor is involved in biological oxidation-reduction reactions, viz. the *catalyst*. In Wieland's experiments colloidal palladium played the part of a combined catalyst and hydrogen acceptor.

Wieland also found that specimens of palladium that had become 'charged' with hydrogen in the manner just described can pass on their hydrogen to certain reducible substances such as the synthetic dye, methylene blue. In the case of methylene

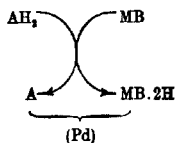
blue itself, reduction yields a colourless substance, leuco-methylene blue, which we may write for the sake of convenience as MB.2H. Thus, in the presence of an oxidizable substance, AH_2 , together with methylene blue, palladium black catalyses two processes, first the dehydrogenation of AH_2 (reaction *a*), and secondly the reduction of methylene blue (reaction *b*):



Hydrogen taken up from the primary hydrogen donator, AH_2 , is passed on to the dye, the catalyst acting as an *intermediary carrier of hydrogen*. This significant fact becomes more apparent if we write the equations in the following unorthodox but very descriptive fashion:



By using this method of expression we can emphasize the essentially *cyclical* manner in which the carrier catalyst acts, a small amount being alternately hydrogenated and dehydrogenated over and over again, and thus participating in a very large amount of chemical change. Alternatively, if we only knew that palladium acts catalytically, but did not know how it does so, we might write the overall process as follows:



The bracket is used here to indicate that (Pd) acts as the catalyst, and the scheme should be interpreted as meaning that AH_2 and methylene blue react together under the catalytic influence of (Pd) with production of A and leuco-methylene blue. We shall use these methods of expression frequently as we go along.

Now living cells and extracts prepared from them by suitable methods are able to oxidize many organic compounds such, for example, as glucose, lactate and succinate. These compounds are quite stable in aqueous solution, and it follows, therefore, that the cells contain enzymes which catalyse their oxidation. The nature and distribution of these enzymes, to which the name

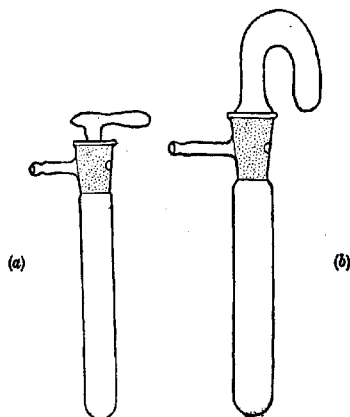


Fig. 13. Thunberg vacuum tubes. (a) Original type, (b) newer type, with hollow stopper. (Drawings by H. Mowl.)

of *dehydrogenases* has been given, were studied extensively by Thunberg, taking advantage of the extreme ease with which methylene blue can be reduced. If methylene blue is added to a suspension of chopped muscle, brain, kidney, etc., or to a suspension of yeast or bacteria, the dye is rapidly reduced, thus revealing the presence in the cells of reducing systems. If such an experiment is tried in the laboratory it is usually found that the dye is decolorized in the bulk of the mixture but not at the surface. This is because leuco-methylene blue is rapidly and spontaneously re-oxidized by atmospheric oxygen. To obviate this difficulty Thunberg introduced the use of vacuum tubes of the type illustrated in Fig. 13, in which is also shown a modern

version in which the simple stopper is replaced by a curved, hollow device. Reagents can be put into the hollow stoppers of these tubes and tipped into the rest of the reaction mixture at any desired moment. Suitable tissue preparations are placed in these tubes, together with buffer, methylene blue and other appropriate reagents, the stoppers are greased and inserted, and the tubes are then evacuated at the pump. When exhaustion has been completed the stopper is turned and the tube is thus sealed. Alternatively, the tubes may be filled with an inert gas, such as hydrogen or nitrogen. In the presence of an active reducing system the methylene blue is reduced, and its reduction is, in effect, an indication of the presence of such a system. This method has been used extensively for studies of tissue respiration. It is quick, very convenient, and has the additional advantage of giving quantitative results, since the time taken to decolorize a given quantity of methylene blue under standard conditions of temperature, pH and so on gives an inverse measure of the activity of the systems concerned. Other dyes such as cresyl blue, pyocyanine, and organic substances such as *m*-dinitrobenzene and *o*-quinone can replace methylene blue.

With the aid of this simple but ingenious technique, Thunberg carried out many important investigations on living tissues. If a sample of minced muscle tissue is placed together with buffer and methylene blue in a vacuum tube, and the latter evacuated, the dye is reduced very rapidly. If now the experiment is repeated using boiled muscle the dye is no longer reduced, indicating that the catalysts concerned are thermolabile and therefore probably enzymes. If a third experiment is performed in which unboiled and unwashed muscle tissue is replaced by tissue that has been minced and well washed, the time taken for the reduction of the dye is much increased. Some part of the complete reducing system has therefore been removed by washing. If, now, substances such as succinate, lactate and the like are added to the mixture of washed tissue, buffer and methylene blue, a rapid reduction of the dye can again be demonstrated. Since these compounds do not reduce methylene blue spontaneously it follows that the tissue must contain enzymes capable of

catalysing their oxidation. Working in this manner Thunberg demonstrated, in cells and tissues of many different kinds, the presence of dehydrogenases catalysing the dehydrogenation of a very wide range of organic materials and, as was later shown by Stephenson, even molecular hydrogen can be oxidized by a bacterial enzyme. Succinate, lactate, citrate, α -glycerophosphate, glucose, aldehydes and alcohols are among the many substances that can be activated by tissue dehydrogenases from one or another source. Some dehydrogenases are relatively uncommon and can only be found in certain tissues, and in such cases their individuality cannot be doubted. Again, the same tissue, worked up in different ways, can yield preparations which catalyse the oxidation of some compounds but not that of others, and by systematic work along these lines a great deal was learned about the specificities of the dehydrogenases.

We know now that there is not one single, master dehydrogenase but a considerable number of different individual dehydrogenases. Some, of which the succinic enzyme is an example, act only upon one substrate, in this case succinic acid; others, such as the aldehyde oxidase (Schardinger enzyme) of milk, catalyse the oxidation of any of a wide range of substrates, in this case aldehydes, aliphatic or aromatic. The dehydrogenases thus show the phenomenon of specificity. They are thermolabile, and their activity is profoundly affected by pH. They are susceptible to the action of many enzyme inhibitors, and often to that of narcotic substances such as the higher alcohols and various substituted ureas. In short, they show all the properties characteristic of enzymes. It was realized fairly early in the history of the dehydrogenases that some members of the group require the co-operation of coenzymes, for after exhaustive washing of the tissues the reduction time for some substrates is very much increased. It can, however, be shortened again by adding boiled extracts of muscle, in which the necessary coenzymes are present. In other cases, however, the addition of boiled muscle-juice does not restore activity, the reason being that some dehydrogenases are themselves soluble in water and therefore removed by vigorous washing of the tissue.

It must be emphasized that the use of methylene blue is not in any sense a natural procedure. The dye does not occur in nature, and it is used simply as a convenient hydrogen acceptor for the visual demonstration of the existence of natural reducing systems. Methylene blue *replaces* the natural hydrogen acceptors of the tissues, and our next inquiries must be into the nature and identity of these substances.

The first likely natural hydrogen acceptor that comes to mind is, of course, molecular oxygen, since it is at the expense of molecular oxygen that tissue oxidations are ultimately carried out. But if we set up experimental systems in which a given dehydrogenase and its substrate are mixed together in the presence of oxygen, we find that of all the known dehydrogenase systems only a few actually take up oxygen. Thus, contrary to all expectation, molecular oxygen is not the natural hydrogen acceptor for most dehydrogenase systems, and we must look further. In the meantime, however, we can distinguish between two groups of dehydrogenases: those which can utilize molecular oxygen as a hydrogen acceptor and are now called *aerobic dehydrogenases*, and the remainder which operate through other hydrogen acceptors and are known as *anaerobic dehydrogenases*.

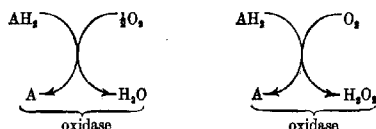
Attention must also be drawn to a third important group of oxidizing enzymes. Like the dehydrogenases in general they catalyse the dehydrogenation of their substrates, but, unlike Thunberg's classical dehydrogenases, cannot reduce methylene blue and similar hydrogen acceptors. Instead they use molecular oxygen, and are therefore known as *aerobic oxidases*. It may be doubted whether their inability to reduce synthetic dyes like methylene blue constitutes adequate grounds for regarding them as essentially different from the aerobic dehydrogenases, and as a matter of convenience they will be considered here along with the aerobic dehydrogenases under the general heading of oxidases.

OXIDASES

The oxidases are distinguished from the other dehydrogenating enzymes by their ability to use molecular oxygen directly as hydrogen acceptor. The aerobic oxidases are specifically confined

to oxygen as a natural acceptor and cannot use other substances, but in the case of the aerobic dehydrogenases, molecular oxygen can be replaced for experimental purposes by methylene blue.

The oxidases may be divided into two groups, the first of which catalyses the reduction of molecular oxygen to water while the second group leads to the formation of hydrogen peroxide. These reactions may be generally expressed as follows:

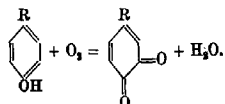


It seems to be characteristic of these enzymes that they are conjugated proteins, although in a few cases no prosthetic group has so far been identified. In general, however, the presence of such a group has been demonstrated and its identity established in a number of cases. Among the known prosthetic materials are copper, iron, perhaps zinc, and adenineflavine dinucleotide.

Phenol Oxidases

The phenol oxidases are a group of enzymes that catalyse the oxidation of phenolic substances. Several representatives of the group have been purified and shown to be copper-containing proteins, thus resembling oxygen-carrying pigments of the haemocyanin type.

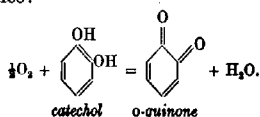
Monophenol oxidase, isolated from mushrooms, catalyses the oxidation of mono-phenols to the corresponding *o*-quinones:



The intimate details of the process are not known. It is perhaps unlikely that the *o*-diphenol is formed as an intermediary product, since this enzyme acts much more strongly upon

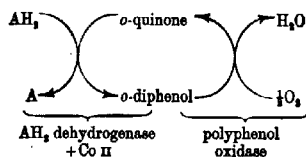
mono- than upon diphenols. Like other oxidases it uses molecular oxygen as hydrogen acceptor but cannot, however, reduce methylene blue. It is, therefore, an aerobic oxidase rather than an aerobic dehydrogenase.

Polyphenol oxidases have been isolated from mushrooms and potatoes and these too are copper compounds. They have no immediate action upon monophenols, but act rapidly upon *o*-diphenols such as catechol to form the corresponding *o*-quinones in the first instance:



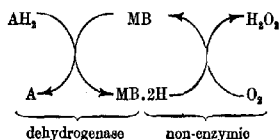
Triphenols such as pyrogallol are also attacked. Another member of this group of phenol oxidases, *laccase*, has been isolated from the latex of the lac tree, and this too is a copper protein, but differs somewhat from the others in specificity.

The primary oxidation is followed as a rule by further changes which are spontaneous, but before we consider these latter, attention may be drawn to one very important feature of the polyphenol oxidase system. The *o*-quinones formed can be rapidly reduced again by ascorbic acid, and by reducing systems such, for example, as hexosemonophosphate dehydrogenase together with its substrate and the appropriate coenzyme (Co II). If we represent the reducing substrate as AH_2 the reactions can be written in the following manner:



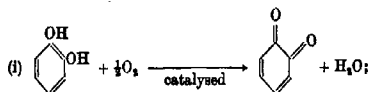
In this system a very small amount of the diphenol, e.g. catechol, or the corresponding quinone can be alternately reduced and oxidized many times and thus can act as an intermediary carrier

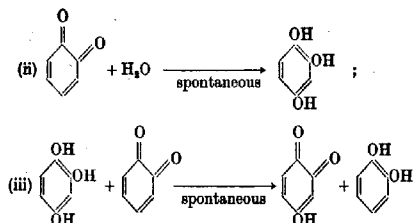
of hydrogen between AH_2 on the one hand and molecular oxygen on the other. Such a system catalyses a continuous uptake of oxygen and a simultaneous oxidation of AH_2 in equivalent amount. The amount of carrier required is very small indeed, and the carrier must, in fact, be regarded as a catalyst in its own right. We have in this system a biological counterpart of the palladium systems studied by Wieland (p. 90), while Thunberg's methylene-blue systems can act in the same way under aerobic conditions:



Systems of this kind are very interesting because they can be regarded as 'models' of the respiratory systems of living cells. These latter contain reducing substances, represented by AH_2 , together with the appropriate dehydrogenases and coenzymes. These pass on hydrogen to acceptors, the identity of which we have to discuss here, and at the other end of the chain molecular oxygen acts as the ultimate hydrogen acceptor, being reduced in the process. Whether systems involving polyphenol oxidase play any important part in the respiration of plant tissues is uncertain, but the possibility does not seem to have been excluded.

Polyphenol oxidase systems are involved in the 'browning' of potatoes, apples and other plant materials that takes place when a cut surface is exposed to the air. It is usually found that plants containing polyphenol oxidase also contain traces of *o*-diphenols, especially of catechol. Catechol, for example, is oxidized to the corresponding *o*-quinone through the agency of the oxidase (reaction (i)) and the remaining stages take place spontaneously:



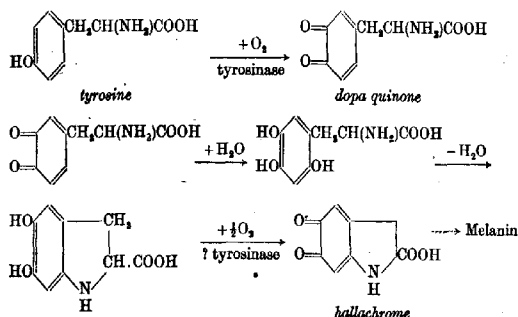


Finally, (iv), the hydroxyquinone undergoes polymerization to yield dark-coloured, melanic products. *o*-Quinone formed in reaction (i) is consumed in reactions (ii) and (iii), and the melanic products can continue to be formed only so long as *o*-quinone is supplied by the action of polyphenol oxidase in reaction (i).

The enzyme *tyrosinase* occurs widely, in plants and animals alike. It is this enzyme that is responsible for the production of most of the dark brown and black pigmentation of animals. In albinism, where there is a characteristic and complete lack of melanins, tyrosinase also is absent. In piebald animals such as rabbits and guinea-pigs the dark portions of the skin contain tyrosinase, but the enzyme is absent from the white parts. In certain insects, on the other hand, melanic pigmentation is determined by localization of the substrate, tyrosine, rather than by that of the enzyme. The dark brown or black 'ink' of the squids and octopuses consists of a fine suspension of the same melanin, which is very insoluble. It is elaborated in a special gland, the ink-sac, the walls of which contain tyrosinase and are at the same time very rich in copper. This is the only indication we have at present that the tyrosinase of animals is a copper protein. Plant tyrosinase has not so far been crystallized in an active form.

The first stage in the formation of melanin from tyrosine probably consists in the oxidation of tyrosine to the quinone of dihydroxyphenylalanine ('dopa quinone'), a reaction that can be catalysed by the monophenol oxidase of plants. This is followed by spontaneous ring closure and by the possibly enzymic oxidation of the product to yield a red pigment called halla-

chrome. Further changes, which are probably spontaneous and include polymerization, give rise to melanin itself:

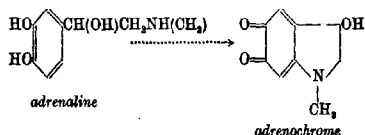


Tyrosinase is involved at perhaps two stages in this reaction sequence, first in the oxidation of tyrosine to dopa quinone, a reaction that can also be catalysed by the monophenol oxidase of plants, and later in the oxidative production of hallachrome from the corresponding *o*-diphenol, a process that can be catalysed by plant polyphenol oxidase.

It may be, therefore, that tyrosinase acts both as a mono- and as a polyphenol oxidase. On the other hand, the oxidative formation of hallachrome from the corresponding diphenol might take place spontaneously at the expense of the reduction of a second molecule of dopa quinone (cf. equation (iii), p. 98), which can be formed from tyrosine by tyrosinase. If so, tyrosinase need only be involved in the capacity of a monophenol oxidase, with the sole function of producing dopa quinone from tyrosine. In this case, however, dopa itself would be formed, and its reoxidation to the quinone would presumably call for polyphenol oxidase activity on the part of tyrosinase. As yet the position has not been satisfactorily cleared up, and we do not know whether tyrosinase is a single enzyme of the monophenol oxidase type, a mixture of a mono- and a polyphenol oxidase, or a single enzyme with the combined activities of phenol oxidases of both types.

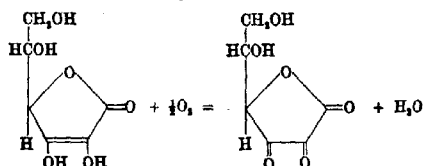
Another interesting transformation that can be catalysed by tyrosinase is the conversion of adrenaline into the physiologically

inactive adrenochrome. The process probably runs parallel to that just described, and the red pigment, adrenochrome, can undergo further changes to yield a dark-coloured melanic material once more:



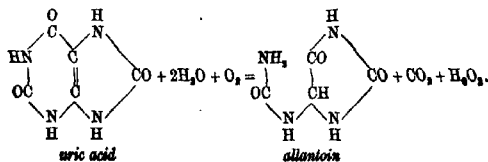
This transformation, too, can be catalysed by the phenol oxidases of plants.

Ascorbic Oxidase. Although ascorbic acid is one of the substances known to be oxidizable through the polyphenol oxidase-catechol system of plants, many plants, notably the *Brassica* family, are rich in ascorbic oxidase. This enzyme catalyses the oxidation of ascorbic to dehydroascorbic acid:



The enzyme has been isolated from squash and shown to contain copper, and it is interesting to notice that a mixture of egg albumin with a trace of copper ions can imitate the action of ascorbic oxidase remarkably closely.

Uric-oxidase (uricase), an enzyme concerned in purine metabolism, catalyses the oxidation of uric acid to allantoin. Its action is almost certainly complex, but the overall reaction may be written as follows:



This enzyme occurs in the livers of animals which are not uricotelic, and is present, for instance, in most mammalian livers. To this, however, there are certain curious exceptions, for man and the higher apes excrete uric acid unchanged, and so too does the Dalmatian dog, though not dogs of other breeds. It is a strange fact that the liver of the Dalmatian dog is nevertheless fairly rich in urico-oxidase.

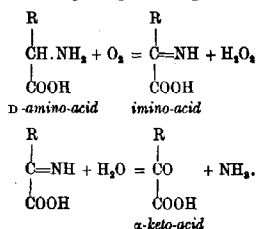
Urico-oxidase is a good deal less specific towards its substrates than is xanthine oxidase, another enzyme of great importance in purine metabolism. It will catalyse the oxidation of several purines other than uric acid, and is competitively inhibited by certain other purines, notably by methylated derivatives of uric acid. It may be that the overall reaction includes spontaneous as well as catalysed processes, as in the case of melanin formation by tyrosinase.

The purest preparations so far obtained contain both iron and zinc. Enzymes containing one or other of these metals are known, but no enzyme so far obtained in the pure state has been found to contain more than one metal, apart, of course, from urico-oxidase itself. This is an example of an aerobic oxidase that produces not water but hydrogen peroxide as the product of reduction of the oxygen used as hydrogen acceptor.

Cytochrome oxidase, a very important and almost universally distributed enzyme, is another example of a metalloprotein, and its prosthetic group, which contains iron, is allied to haem. We shall return to discuss this enzyme in greater detail. It is another example of an aerobic oxidase.

D-Amino-acid oxidase, unlike the oxidases so far discussed, is able to utilize methylene blue as hydrogen acceptor although its natural acceptor is molecular oxygen, which is reduced to hydrogen peroxide in this case. This enzyme occurs in the liver and kidney tissue of mammals, and is probably present in many other animal materials. It is a curious fact that this enzyme acts only upon members of the non-natural D-series of amino-acids and is without action upon the L-acids. As far as the D-acids are concerned it is group-specific and attacks them all with a few exceptions, e.g. D-glutamic acid.

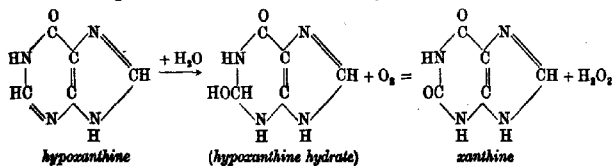
D-Amino-acid oxidase catalyses the oxidative deamination of its substrates to the corresponding α -keto-acids. This takes place in two stages, of which the first, consisting in the dehydrogenation of the substrate, is catalysed, while the second, in which the resulting imino-acid is hydrolysed, is probably spontaneous:

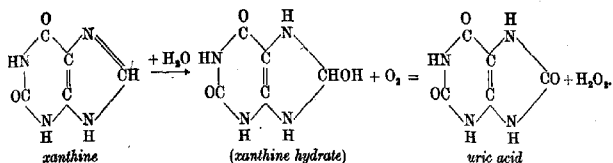


As far as is known, D-amino-acids occur only rarely in nature, and it may therefore be doubted whether this enzyme has any important biological function. That it exists, however, is certain, and it has in fact been isolated. It proves to be a conjugated protein, the prosthetic group of which is adenineflavine dinucleotide, the mode of action of which is known and will be discussed presently.

Other deaminating oxidases include a specific D-glutamic acid oxidase, a specific glycine oxidase, and a group-specific L-amino-acid oxidase.

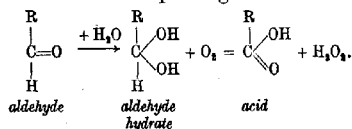
Xanthine oxidase is present in milk, which offers a fairly rich source from which highly concentrated preparations can fairly easily be obtained. The enzyme is widely though somewhat erratically distributed among animal tissues. It catalyses the oxidation of hypoxanthine to xanthine, and that of xanthine to uric acid: here, as in the oxidation of aldehydes by dehydrogenation, it is probable that intermediate hydrates are formed:





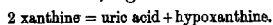
The specificity of xanthine oxidase towards purines is very high. Its action has been tested upon numerous purines and related substances, but, apart from hypoxanthine and xanthine, no naturally occurring purines are attacked at all rapidly. The enzyme does, however, act upon certain synthetic substances such as 6:8-dioxypurine and upon purine itself, while it is strongly and competitively inhibited by adenine.

In addition to its action upon the purines, xanthine oxidase has a second sphere of action. It is identical with the Schardinger enzyme of milk, a catalyst which acts upon many aldehydes, oxidizing them to the corresponding acids:



Xanthine oxidase has been highly concentrated and, like D-amino-acid oxidase, the purified preparations contain adenine-flavine dinucleotide. It is probable, however, that some other prosthetic material is also present, and it is even possible that the supposedly pure preparations are in reality mixtures, since their catalytic action differs somewhat according to the method of preparation.

In addition to catalysing the various oxidations already described, xanthine oxidase can catalyse a process of *dismutation*, i.e. the oxidation of one molecule of the substrate at the expense of the reduction of another, e.g.

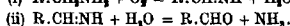
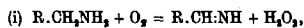


Dismutation only occurs under anaerobic conditions: in the absence of molecular oxygen, the same enzyme can utilize a second molecule of its substrate as hydrogen acceptor, and a pair of

hydrogen atoms is transferred from one molecule to a second. Reactions of this kind play an important part in cellular metabolism under anaerobic conditions, and they resemble the well-known Cannizzaro reaction of organic chemistry in certain respects.

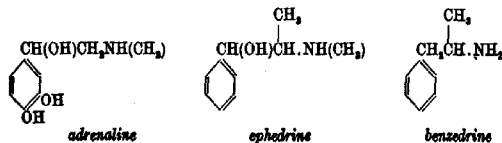
Aldehyde oxidase of liver. Mammalian liver contains another aldehyde oxidase. This enzyme, like xanthine oxidase, is group-specific towards aldehydic substrates, which it oxidizes to the corresponding acids, but, unlike xanthine oxidase, it has no activity towards purines.

Amine oxidase is a group-specific enzyme that occurs at small concentrations in many animal tissues. It acts particularly rapidly upon compounds such as tyramine and adrenaline, and appears to be identical with the tyramine and adrenaline oxidases described some years ago. Its action upon primary amines may be described as follows:



The mode of oxidation thus resembles that of the amino-acid oxidases somewhat, and it is probable that the second reaction is not catalysed but spontaneous. The enzyme also acts upon secondary and tertiary amines to form either a lower amine, or else an aldehyde together with ammonia.

An interesting point to notice here is that, while this enzyme acts upon many amines, it is inhibited by those which contain a methyl group in the α -position, for instance by ephedrine and benzedrine. Adrenaline is normally destroyed in the tissues by amine oxidase, in part at least, and its disappearance is much retarded by these drugs. Probably, therefore, these compounds owe their adrenaline-like action upon the organism to an indirect action which prevents the oxidation of adrenaline itself, rather than to a direct action upon the tissues. The structural relationships between these substances are as follows:



Diamine oxidase also occurs in animal tissues. It catalyses the oxidation of histamine, putrescine, cadaverine and agmatine, for example, and these substances can arise in the gut of animals by bacterial action. It is probable that both the amine oxidases are important in the oxidative detoxication of poisonous amines formed in the intestine by bacterial decarboxylation of amino-acids.

The fate of Hydrogen Peroxide

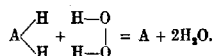
The formation of hydrogen peroxide in the course of the *in vitro* action of certain oxidases may be demonstrated by taking advantage of the fact that cerous hydroxide, a colourless substance only slightly soluble in water, reacts readily with hydrogen peroxide to form an insoluble, yellowish brown ceric peroxide. While there can be no doubt that hydrogen peroxide is indeed formed in isolated enzyme systems, there is little evidence that it is formed, or at any rate that it ever accumulates to any extent, in cells and tissues generally. We must therefore conclude that living cells contain catalysts capable of destroying hydrogen peroxide which, as is well known, is a powerful inhibitor of many enzymes. At least three enzymes are known to participate in its destruction, namely, cytochrome c-peroxidase, peroxidase and catalase.

Cytochrome c-peroxidase is probably responsible for the removal of most of the hydrogen peroxide formed in living cells, but unlike other peroxidases it is absolutely specific towards the substrate of oxidation, in this case the reduced form of cytochrome c. This is oxidized to the ferric form, the peroxide being at the same time reduced to water.

Peroxidase occurs in many plants, often in relatively high concentration. Animal tissues in general appear not to contain enzymes of this kind, though a peroxidase has recently been isolated in crystalline form from milk. Horse-radish peroxidase has been shown to be a haematin compound.

Peroxidase forms a spectroscopically recognizable complex with hydrogen peroxide, as a result of which the substrate becomes activated and capable of acting as a hydrogen acceptor

for the oxidation of other substances. The classical test for peroxidase consists in adding to the suspected enzyme small amounts of hydrogen peroxide, together with guaiacum or benzidine. If peroxidase is present a characteristic deep coloration develops owing to the oxidation of the guaiacum or benzidine to yield a coloured derivative. Pyrogallol is similarly oxidized through a series of intermediates to form the orange-coloured purpurogallin, which can be extracted with ether and estimated colorimetrically, a process which has been made the basis of a method for the quantitative estimation of peroxidase activity. The behaviour of the peroxidase-peroxide system can be generally expressed as follows:



It is interesting to notice in passing that most haem and haematin derivatives, including even free haematin, possess a feeble peroxidase activity.

Catalase occurs both in animals and plants. It has been isolated from mammalian liver and found to be a conjugated protein, of which the prosthetic group can exist in an oxidized or a reduced form, the latter being identical with the haem of haemoglobin. Catalase catalyses the splitting of hydrogen peroxide into water and molecular oxygen and, according to Keilin and Hartree, who have studied this enzyme with great thoroughness, the iron atom of the prosthetic group undergoes alternate oxidation and reduction in the process. Catalase forms spectroscopically recognizable compounds with a number of substances other than hydrogen peroxide, including cyanide, hydrogen sulphide, azide and fluoride, all of which inhibit it. Cyanide and sulphide react with the ferric form of the enzyme while azide prevents reoxidation of the ferrous form.

Catalase appears to be very specific, for it does not act upon organic peroxides such as ethyl peroxide except in very concentrated solutions. Its action upon hydrogen peroxide is very rapid however, and it has been shown that 1 mg. of pure catalase-iron produces 2740 l. of oxygen per hour from hydrogen peroxide,

at 0° C. Expressed in molecular units this corresponds to the decomposition of 4.2×10^4 mol. of hydrogen peroxide per sec. at 0° C. by 1 mol. of catalase. This is the most rapid enzyme-catalysed process at present known.

Mode of Action of the Oxidases

We saw in an earlier section that in the oxidation of biological substances three main factors are involved, viz. the hydrogen donor, the hydrogen acceptor and the catalyst. In a typical oxidase system these are represented by the substrate, molecular oxygen and the oxidase respectively. The system can be analysed a little further, however, for in several cases it has been shown that the prosthetic group participates in the reaction and undergoes alternate oxidation and reduction. As a specific case we may consider the oxidation of a D-amino-acid by D-amino-acid oxidase. In this case the prosthetic group consists of adenineflavine dinucleotide, a substance which shows a well-defined absorption in the blue region of the visible spectrum. If a D-amino-acid is added to the oxidase under strictly anaerobic conditions, the spectrum changes in a manner which indicates that the prosthetic group has undergone reduction. If oxygen is then readmitted to the system, the prosthetic group is reoxidized.

Now it is known that adenineflavine dinucleotide can be reduced by fairly powerful reducing agents, even when it is in the free condition and apart from its specific protein partner. It is not, however, reduced by amino-acids unless and until they have been activated by the oxidase. It is therefore reasonable to suppose that the oxidase protein acts as a catalyst which facilitates the transference of hydrogen from the (activated) amino-acid to the dinucleotide. Probably, however, the protein plays no part in the second stage of the process, in which the reduced dinucleotide is reoxidized by molecular oxygen, for uncombined adenineflavine dinucleotide is spontaneously oxidized by oxygen. Tentatively, then, we may picture the action of this oxidase as a two-stage process, the protein component acting as a catalyst for the first stage, while the prosthetic

tion of oxygen. His attention was drawn by the fact that iron compounds are capable of catalysing the oxidation of many different organic compounds. Blood charcoal, i.e. charcoal prepared by heating blood, catalyses the oxidation of organic substances such as the amino-acids with a simultaneous uptake of oxygen. Pure charcoal made by heating sucrose does not possess this property, and Warburg attributed the catalytic action of blood charcoal to the iron it contains. Even in inorganic form, iron possesses considerable catalytic power, but Warburg came early to the conclusion that in organic combination with nitrogenous substances its catalytic powers can be very great indeed.

The behaviour of blood-charcoal systems resembles that of living cells to a surprising degree. The oxygen uptake of these charcoal 'models' is powerfully inhibited by a number of substances known to combine with heavy metals and to inhibit cellular respiration. Thus cyanide at concentrations of the order of $m/1000$ suffices to stop 80-90% of the total respiration of many cells, and cyanide at similar concentrations also inhibits charcoal systems very powerfully indeed. Hydrogen sulphide acts similarly. Narcotic drugs, such as urethane and the higher alcohols, also inhibit cellular respiration, but do so only at comparatively high concentrations, and they act in the same general manner upon charcoal systems as well. Warburg was led therefore to the idea of a universal, iron-containing catalyst concerned in cellular respiration, to which he gave the name of *Atmungsferment*, or respiratory enzyme. It is not necessary here to go into Warburg's work in great detail, but some at least of his evidence is of fundamental importance.

Careful attention was given to certain processes of autoxidation, notably in the case of cysteine. Cysteine undergoes slow autoxidation to cystine on exposure to the air, and the process is known to be catalysed by traces of iron-containing impurities. Cyanide stops this autoxidation, presumably by forming a non-catalytic complex with the iron. The fact that molecular oxygen no longer oxidizes cysteine in the absence of iron lent valuable support to Warburg's contention that it is an essential function

of iron compounds to catalyse oxidative processes by activating molecular oxygen.

Most striking of all were the results of certain experiments on the inhibitory effects of carbon monoxide. Warburg found

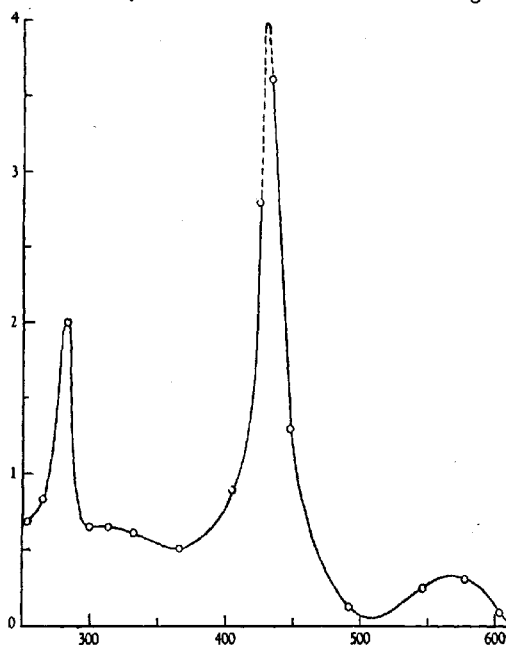


Fig. 14. Absorption spectrum of the carbon monoxide compound of the *Atmungsferment*. Ordinate: $\beta \times 10^4$. Abscissa: λ . (Plotted from data of Warburg & Negelein, 1929.)

that the respiration of living cells is inhibited by carbon monoxide in the dark, but that the inhibition disappears in the light. Carbon monoxide is known to form complexes with heavy metals, including iron and copper for example, and it is characteristic of the iron complexes that they are photolabile, i.e. are dissociated by light. The copper complexes, by contrast, are not

influenced by light. Here, therefore, was further evidence that the respiratory enzyme must contain iron.

Now the effectiveness of light in reversing the inhibition produced by carbon monoxide depends upon the wave-length of the light. In order to be effective, light must be absorbed.

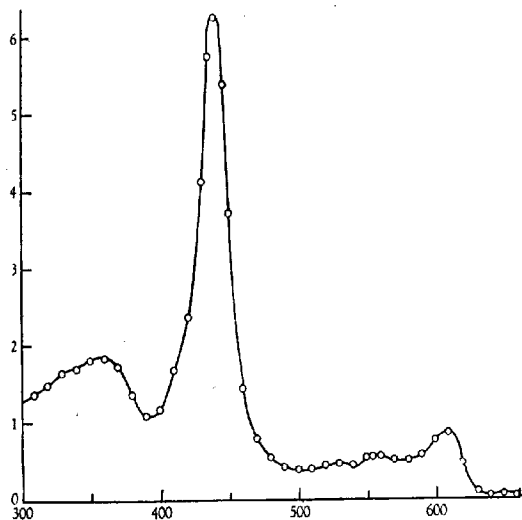


Fig. 15. Absorption spectrum of a haemochromogen (carbon monoxide compound of a chlorocruorin). Other haemochromogens give spectra of a similar pattern but with the bands in different positions. Ordinate: $\beta \times 10^4$. Abscissa: λ . (Plotted from data of Warburg, Negelein & Haas, 1930.)

Consequently, by plotting the effectiveness of light against its wave-length it is possible to determine the absorption spectrum of the respiratory enzyme. The resulting curve is reproduced in Fig. 14. It shows a sharp band at 4360 Å., and the general pattern resembles that of the haemochromogens (cf. Fig. 15). Further work enabled Warburg to determine the absolute absorption coefficient of the respiratory enzyme, and this again was in general agreement with values obtained for haemochromogens. Thus, without having ever seen his enzyme, Warburg was

able to deduce not merely that it contains iron, but that it must be a haemochromogen-like material. A great mass of other work gave general support to his conclusions.

In the meantime Keilin rediscovered the presence, in aerobic cells of many different kinds, of a pigment which had been described by MacMunn towards the end of the last century. MacMunn had called his pigment histohaematin, but it was soon forgotten. Keilin rechristened it *cytochrome*. Cytochrome, he found, was present in every kind of aerobic cell he examined, even in facultative anaerobic bacteria: indeed, the only cells in which none could be detected were strictly anaerobic bacteria. There was, moreover, a general parallel between the respiratory activities of different tissues and the amounts of cytochrome they contained.



Fig. 16. Absorption spectrum of cytochrome in the thoracic muscles of a bee. (After Keilin, 1925.)

Cytochrome is clearly recognizable by the well-defined absorption spectrum which it exhibits when in the reduced form. In the oxidized form the spectrum disappears. Slight differences were noticed in the positions of the absorption bands in different tissues, but, on the whole, these differences were not greater than would be expected if the cytochromes, like the haemoglobins, were species-specific. Fig. 16 shows the absorption spectrum of reduced cytochrome as seen in the thoracic muscles of a bee, while Fig. 17 shows the positions of the four absorption bands of the reduced cytochromes of a number of different materials. That one or other of these bands is occasionally found to be absent, and that they vary somewhat in relative intensity, shows that cytochrome is not a single substance but a mixture of at least three components, each being a haemochromogen-like compound with a typical two-banded spectrum. Fig. 18 shows how the spectrum of a typical cytochrome could be built up by

the superposition of the spectra of three compounds of this type.

A particularly attractive feature of much of Keilin's work is that it was possible to observe the behaviour of cytochrome within the living cell when the latter was subjected to a variety

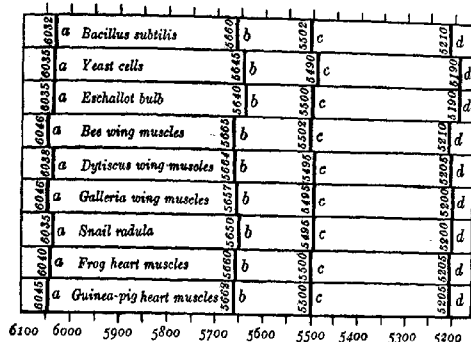


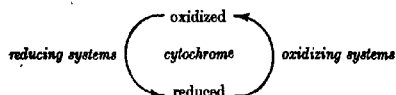
Fig. 17. Absorption bands of cytochromes of various tissues.
(After Keilin, 1933.)

	α	b	c	d
Cytochrome	α_1	α_2	α_3	$\beta_1, \beta_2, \beta_3$
Compound α'	α_1			β_1
" b'		α_2		β_2
" c'			α_3	β_3
Red				
Blue				

Fig. 18. Absorption bands of the components of a typical cytochrome.
(After Keilin, 1933.)

of experimental conditions. Certain of these experiments were of fundamental importance. A suspension of bakers' yeast shows no spectrum of cytochrome so long as oxygen is bubbled through it. If the stream of oxygen is replaced by nitrogen the spectrum of reduced cytochrome makes its appearance, and persists until oxygen is again admitted. These simple observations alone show

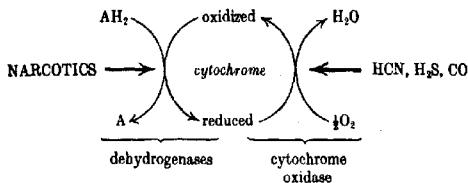
that the cells contain systems which can reduce oxidized cytochrome and others which can reoxidize it in the presence of oxygen. Similar observations on other cells show that the existence of systems of the same kind is widespread. Thus we may write a scheme such as the following:



Keilin now examined the effects of respiratory inhibitors upon the behaviour of cytochrome, and found that poisons such as cyanide, hydrogen sulphide and carbon monoxide prevent the oxidation of reduced cytochrome. Narcotics, on the other hand, prevent the reduction of oxidized cytochrome. The effects of all these inhibitors can readily be studied in suspensions of yeast with apparatus no more complicated than a test-tube and a suitable spectroscope. The results just outlined indicate the presence in the cells of systems which oxidize reduced cytochrome and are inhibited by cyanide, carbon monoxide, etc., and of reducing systems that are inhibited by narcotics.

A few years later Keilin succeeded in isolating the *c* component of cytochrome from bakers' yeast. The pigment is readily reduced by mild reducing agents, but, once reduced, cannot be reoxidized merely by shaking with oxygen. Yet it is reoxidized readily enough when oxygen is admitted to a cell suspension. This argues for the presence in the cells of an enzyme which may be called *cytochrome oxidase*. The cytochrome oxidase of living cells is insoluble and cannot be separated from the cell debris,⁴ but can be studied in the form of thoroughly washed tissue suspensions. It proves to be powerfully inhibited by cyanide, by hydrogen sulphide and by carbon monoxide. Further, as Keilin himself showed, oxidized cytochrome can be reduced by the dehydrogenase systems of Thunberg, e.g. by succinic dehydrogenase together with succinic acid. These dehydrogenase systems are known to be inhibited in many cases by narcotics, and are evidently responsible for the intracellular reduction of

cytochrome, the systems for which are, as Keilin had shown, sensitive to the same narcotics. We may therefore summarize the position in the following manner, using heavy arrows to indicate the reactions with which the various inhibitory substances interfere:



It would thus seem that we have in cytochrome the natural hydrogen acceptor of the dehydrogenase systems. Clearly, therefore, it is necessary to know something about its various components. Most is known about cytochrome *c*, which is the only soluble member of the group, the others remaining closely attached to the tissue debris. Methods have recently been devised for detaching the other components, but we do not yet possess a great deal of information about them. The only method hitherto available for their study consisted of the spectroscopic observation of their behaviour in very finely divided and exhaustively washed tissue suspensions. Heart muscle provides a relatively rich source of these materials.

Cytochrome c has been found to consist of a protein carrying an iron-containing prosthetic group. The latter is allied to; but not identical with, the haem of haemoglobin and is attached more firmly, and in a different manner, to its protein partner. This component is readily reduced by fairly mild reducing agents, including biological materials such as cysteine, and by relatively crude dehydrogenase preparations in the presence of their substrates. For its oxidation cytochrome oxidase is required, and the process is inhibited by respiratory inhibitors of the cyanide group.

Cytochrome b, like *c*, is a conjugated protein of which the prosthetic group, so far as we know, is identical with that of

haemoglobin. It, too, is reducible by dehydrogenase systems, but differs from *c* in that it is slowly autoxidizable, i.e. it can be oxidized by molecular oxygen without the intervention of the oxidase. Its oxidation is therefore not totally abolished by cyanide, but there is a marked inhibition nevertheless, since *b* is oxidized more rapidly by oxygen in the presence than in the absence of the oxidase.

Cytochrome a has been found by very careful spectroscopic study to consist of two components, now called *a* and *a₃*. Both are haemochromogen-like substances, the haem of which differs from that of haemoglobin but closely resembles that of chlorocruorin, an oxygen-carrying pigment which is found only in the bloods of certain annelid worms. Component *a* resembles *c* in that it is not autoxidizable and can only be oxidized through the agency of cytochrome oxidase, while *a₃*, which is autoxidizable, differs from all the rest in forming a compound with carbon monoxide, as does haemoglobin.

Occasionally *other cytochrome components* are found. In many bacteria, for example, we find new components which differ from those of animal tissues and have been called *a₁* and *a₂*. They are uniquely confined to bacteria. Special cytochromes characteristic of plants have also been described and, in all, about ten different cytochromes have now been recognized. Of particular interest is the recent discovery by Bach and Dixon that yeast contains a cytochrome, the spectrum of which resembles that of *b* but is not identical with it. In the intact cell the spectrum of this substance, to which the title of *b₂* has been given, is not observable because it is too dilute, but the pigment has been separated, highly concentrated, and shown to form a part of the lactic dehydrogenase system of yeast.

The mechanism of the oxidation and reduction of the cytochromes is not certainly known. The only observable difference between the oxidized and reduced forms of *c* lies in the valency of the iron atom, which passes with oxidation from the ferrous to the ferric condition, a change which is analogous to that which takes place when haemoglobin is oxidized to methaemoglobin.

Identity of Cytochrome Oxidase

Oxidized cytochrome is readily reduced by many mild reducing agents such as cysteine, *p*-phenylene diamine, hydroquinone and the like. Keilin studied the behaviour of cytochrome oxidase in reconstructed systems in which the natural reducing systems of the cell were replaced by cysteine, while cytochrome was represented by the *c* component. The oxidase preparation employed consisted of a finely divided and exhaustively washed suspension of heart muscle.

It had already been known for many years that most living cells and tissues contain an enzyme which catalyses the oxidative coupling of a mixture of α -naphthol with dimethyl-*p*-phenylene diamine, the so-called 'Nadi' reagent, to yield indophenol blue. This enzyme, the natural substrate of which was then unknown, was called *indophenol oxidase*. Keilin found that preparations of indophenol oxidase will catalyse the oxidation of cytochrome *c*, and that preparations of cytochrome oxidase will catalyse the oxidation of 'Nadi'. Furthermore, both enzymes are similarly distributed in living organisms, both are closely bound to the tissue substance, and both are insoluble. Both are powerfully inhibited by cyanide and by hydrogen sulphide, while both are also inhibited by carbon monoxide in the dark but not in the light. Keilin therefore concluded that indophenol oxidase and cytochrome oxidase are one and the same enzyme.

The reader will have noticed that the properties of cytochrome oxidase are identical with those of Warburg's respiratory enzyme, even in the effect of light upon the inhibition produced by carbon monoxide. The resemblances go even further, for both are apparently universally distributed in living cells, and both, while exceedingly sensitive to cyanide and the like, are relatively insensitive to narcotics. Keilin therefore suggested that Warburg's respiratory enzyme and cytochrome oxidase must be identical.

The most recent contribution comes from Melnick, who determined the photochemical absorption spectrum of the carbon monoxide compound of cytochrome oxidase, and obtained the

results shown in Fig. 19. The general form of the curve closely resembles that obtained by Warburg in his classical work on the respiratory enzyme, and the main peak occurs at a similar point in both cases. Warburg's experiments were performed on yeast and on acetic acid bacteria, while Melnick used heart muscle. The fact that there are distinct differences between the two may

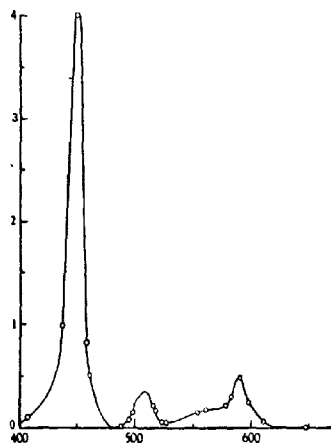


Fig. 19. Absorption spectrum of the carbon monoxide compound of cytochrome oxidase (heart). Ordinate: $\beta_{\lambda}/\beta_{420}$. Abscissa: λ . (Plotted from data of Melnick, 1942.)

reasonably be attributed to species-specific differences between the different materials, and it seems difficult to escape the conclusion that cytochrome oxidase and the respiratory enzyme are truly one and the same catalyst. Even if, as some believe, the systems studied by Warburg on the one hand and by Keilin and later by Melnick on the other, are not absolutely identical, it is still true that Melnick's results demonstrate that the cytochrome oxidase activity of heart muscle is associated with a haemochromogen-like, and therefore an iron-containing catalyst, just as did Warburg's work in the case of the respiratory enzyme of yeast and of acetic acid bacteria. Probably, therefore, we are

justified in concluding, with Keilin, that the respiratory enzyme is none other than cytochrome oxidase, provided we allow for the possibility of species-specific differences between the protein components in different organisms.

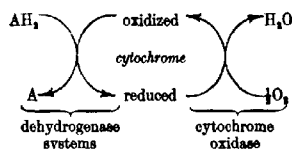
That cytochrome oxidase appears to be a haemochromogen-like compound suggests that it might actually correspond to one of the numerous known components of cytochrome itself, and this does in fact seem possible. Keilin and Hartree have shown that there is a close correspondence between the properties of cytochrome a_3 and cytochrome oxidase. Both form a compound with carbon monoxide, and the absorption curve of the CO-cytochrome a_3 complex shows maxima at 5900 and 4300 Å. The carbon monoxide compound of the respiratory enzyme of yeast shows almost identical maxima. Nevertheless, the identity of cytochrome oxidase with the a_3 component of cytochrome cannot yet be accepted as proven, if only because the carbon monoxide complex of a_3 seems to be stable in the presence of light, and because some cells appear to contain neither a nor a_3 . Further, there is the serious objection that, if reduced cytochrome c is added to tissue preparations containing oxidized a_3 , the two fail to react together.

To sum up we may say that aerobic cells of many different kinds have been found to contain cytochrome components, usually a , a_3 , b and c . Of these c not infrequently occurs in the greatest concentrations, and is probably therefore of the greatest quantitative importance. Component b is autoxidizable, if only slowly, but a and c by contrast require the presence of cytochrome oxidase for their oxidation by molecular oxygen. This enzyme is identical with indophenol oxidase and with Warburg's respiratory enzyme, and may possibly be identical with cytochrome a_3 itself. All these substances occur only at small concentrations in living cells.

The cytochromes can be reduced by dehydrogenase systems and oxidized by molecular oxygen in the presence of cytochrome oxidase. If the oxidase is put out of action by the addition of $m/1000$ cyanide, about 80–90% of the total respiration of many cells and tissues is abolished. It is probable, therefore, that the

bulk of the whole respiration of these tissues goes on by way of cytochromes *a* and *c*. The oxidation of component *b* is slowed but not stopped by cyanide, and this component may therefore be responsible for a part at least of that fraction of the total respiration that is not abolished by cyanide.

It seems, then, that in the cytochromes we have a group of hydrogen carriers of enormous importance in cellular respiration. On the one hand they are reduced from the ferric to the ferrous condition by reacting with hydrogen drawn from the activated substrates of the dehydrogenases, and on the other they are oxidized by molecular oxygen. By repeated alternate oxidation and reduction, small amounts of these cytochromes are able to participate in the oxidation of relatively enormous amounts of material in living cells: their action is, in fact, that of carriers. The action of the cytochromes may be collectively written in the following manner, with the reservation that, in the case of cytochrome *b*, no oxidase is required to catalyse the reoxidation of the reduced material, though its reoxidation is faster when the oxidase is active than when it is inhibited:



DEHYDROGENASES AND CO-DEHYDROGENASES

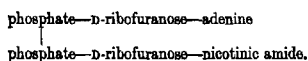
We have already discussed some of the early work on the dehydrogenases, much of which was carried out by the method devised by Thunberg. An essential part of this procedure consists in washing the tissue thoroughly. It happens that some dehydrogenases are water-soluble, and any such would of course be removed by exhaustive washing. Quite apart from these, however, many of the insoluble dehydrogenases lose their power to catalyse the reduction of methylene blue as a result of thorough washing or prolonged dialysis. In such cases the activity is regained if a little boiled tissue juice is added, and

it follows, therefore, that activators or coenzymes of some kind are required, and that the appropriate co-substances are present in the tissues.

The first attempt to isolate such a coenzyme was made by Szent-Györgyi, who obtained a compound which contained adenylic acid and functioned as a coenzyme with respect to the lactic dehydrogenase of heart muscle. Unfortunately, he was unable to characterize his product more fully. A little later Warburg and Christian showed that the hexosemonophosphate dehydrogenase of red blood corpuscles also requires a coenzyme, and set themselves the task of isolating it. Starting with some 250 l. of horse blood they obtained about 20 mg. of a highly purified product. In the meantime, much interest had attached to the coenzymes of yeast juice, one of which was isolated by Euler. On analysis it appeared that these two coenzymes are closely similar in composition, since the following substances were obtained by hydrolysis:

Molecules of	Warburg and Christian's coenzyme = Co II	Euler's yeast coenzyme = Co I
Phosphoric acid	3	2
D-Ribose	2	2
Adenine	1	1
Nicotinic amide	1	1

The constitution of Co I is believed to be that of an adenine-nicotinic amide dinucleotide:



The position taken up by the third phosphate radical in Co II has not been certainly established: many are of the opinion that it is interposed between the other two.

The behaviour of these two coenzymes has been studied by taking advantage of the fact that they show strong absorption bands in the ultra-violet (Fig. 20). In the oxidized form they show a strong, single band at about 2600 Å., while in the reduced form the height of this band is somewhat reduced, and a second band, this time a rather broad one at about 3400 Å., makes its appearance. The sharp band at 2600 Å. can be accounted for by

the known absorption spectra of adenine and nicotinic amide, but the new band at 3400 Å., which appears only in the reduced forms of the coenzymes, can be accounted for by neither. It is due, as Warburg was able to show, to the reduction of the pyridine ring.

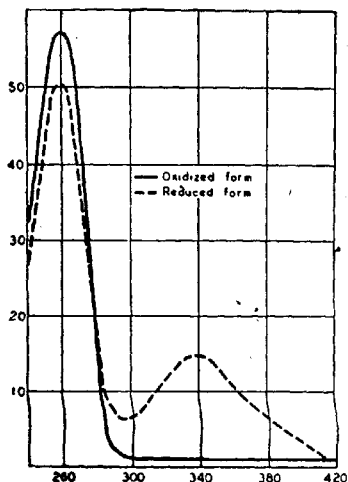
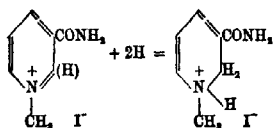
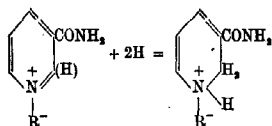


Fig. 20. Absorption spectra of oxidized and reduced Co I.
(After Schlenk, 1942.)

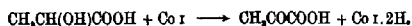
Working with the methiodide of nicotinic amide, he found that reduction leads to the appearance of a new band at 3600 Å., so that the 3400 Å. band of the reduced coenzymes must probably be due to the presence of a reduced pyridine ring: in other words, the oxidation and reduction of the coenzymes as a whole must probably take place at the pyridine ring. The reduction of nicotinic amide methiodide takes place as follows:



It is believed that the nicotinic amide radical of the coenzymes is linked to the sugar radical through its ring-bound nitrogen, and the reduction of the oxidized to the reduced form of either coenzyme is therefore believed to take place as follows, where R^- represents the remainder of the molecule, which is negatively charged through the ionization of one of its phosphoric acid radicals:

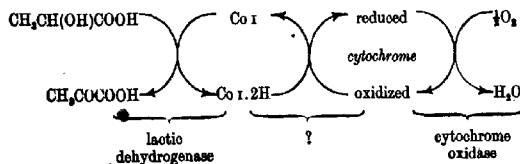


The functional behaviour of Co I can be studied by adding lactic acid and lactic dehydrogenase, for example, to a solution of the oxidized form of the coenzyme in the presence of cyanide, when the following reaction takes place:



This particular system tends towards an equilibrium that is very much in favour of lactic acid, and cyanide is added because it forms a cyanhydrin with the pyruvic acid so that the reaction is pushed over towards the right. The reduction of the coenzyme can be followed by observing the development of the characteristic absorption band at 3400 Å.

Clearly, therefore, the coenzyme works by acting as a hydrogen acceptor for the lactic acid-lactic dehydrogenase system. If to this system we add Co I and cytochrome *c*, the latter is reduced, provided the dehydrogenase has not been exhaustively purified, and by the further addition of cytochrome oxidase it is possible to build up the following system:



The intermediate stage marked ? also requires a catalyst, the nature of which will be discussed later, but this is present in the tissues and in any fairly impure dehydrogenase preparations extracted from them.

This system, taken as a whole, behaves in a manner parallel to that of an intact cell except, of course, that it will only oxidize lactic acid. Nevertheless, it is affected in the same manner and to the same extent as an intact cell by respiratory inhibitors such as narcotics on the one hand and by cyanide, carbon monoxide, etc., on the other. Lactic acid undergoes continuous oxidation in this system, and its oxidation is attended by the uptake of an exactly equivalent amount of oxygen.

With the isolation of the two coenzymes it has become possible to classify dehydrogenases under three headings. Some can reduce methylene blue and cytochrome without the addition of any coenzyme. A second group can only do this in the presence of Co I, while a third group requires the presence of Co II. We have already seen that dehydrogenases are as specific towards their substrates as are other enzymes, such, for instance, as the glycosidases. We now know that they are specific also with respect to their hydrogen acceptors. Lactic dehydrogenase obtained from muscle, for instance, normally requires Co I and cannot utilize the closely similar Co II, while hexosemonophosphate dehydrogenase requires Co II and cannot collaborate with Co I.

But the relationships between the dehydrogenases and their substrates are more specific than those between the dehydrogenases and their coenzymes, for whereas the same coenzyme can collaborate with more than one dehydrogenase, a given substrate is activated only by the appropriate dehydrogenase and not by any other. A further point to be noticed is that the coenzyme itself is not a very reactive compound, for it can only be reduced by comparatively powerful reducing agents such as dithionite (hydrosulphite). Furthermore, lactic acid is not easily oxidized, and becomes easily oxidized only as a result of its union with the lactic enzyme and consequent activation. Similarly, Co I undergoes a great increase in chemical reactivity in

the presence of lactic dehydrogenase, with which it is able to combine. The functional behaviour of the coenzyme is therefore very similar indeed to that of the substrate, and the coenzyme might even be regarded as a second substrate. Both combine with the dehydrogenase protein, and the affinity constants have been determined for both cases.

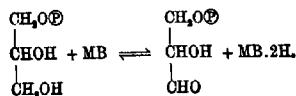
For convenience of reference a list of the most important dehydrogenases is given in Table 5, so as to show which coenzyme, if any, is required by which dehydrogenase. In what follows we shall refer to these groups as *cytochrome-specific*, *Co I-specific* and *Co II-specific dehydrogenases* respectively. In the presence of their substrates and the appropriate coenzymes, all these dehydrogenases are capable of catalysing the reduction of methylene blue and of cytochrome, providing always that they have not been rigorously purified beforehand. We shall now consider the dehydrogenases individually and in more detail.

TABLE 5. COENZYME REQUIREMENTS OF DEHYDROGENASES

Co-dehydrogenase required		
None	Co I	Co II
α -Glycerophosphate ¹	α -Glycerophosphate ¹	Glucose
Succinic	Lactic (muscle)	L-Glutamic
Lactic (yeast)	Malic	Hexosemonophosphate
	Triosephosphate	iso-Citric
	Alcohol	
	β -Hydroxybutyric	
	Glucose	
	L-Glutamic	
	¹ Insoluble.	² Soluble.

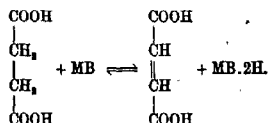
Dehydrogenases requiring no coenzyme

α -Glycerophosphate dehydrogenase (insoluble) catalyses the oxidation of L-(+)- α -glycerophosphate to 3-phosphoglyceraldehyde blue or cytochrome c:

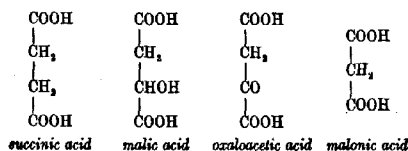


This enzyme is specific for the L-(+)-isomer of α -glycerophosphate: the D-(-)-form is not attacked, nor is either of the β -glycerophosphates.

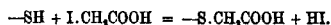
Succinic dehydrogenase appears to be almost universally distributed and catalyses the oxidation of succinic to fumaric acid:



The enzyme is very specific. Apart from succinic acid it is only known to attack dimethylsuccinic acid, and is strongly and competitively inhibited by a number of other dibasic acids, notably by malonic, malic and oxaloacetic acids. As their formulae show, these resemble succinic acid rather closely and so are able to combine with the active groups of the enzyme:



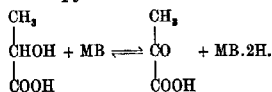
The activity of this enzyme depends upon the presence of its —SH groups, and it is inhibited, this time non-competitively, by agents which oxidize adjacent pairs of —SH groups to form —S—S— linkages. It is also inhibited by fairly high concentrations of moniodoacetate, which reacts with and blocks the —SH groups irreversibly:



Several other dehydrogenases are similarly affected by iodoacetate, but the succinic enzyme requires unusually high concentrations for its effective inhibition.

Lactic dehydrogenase of yeast. This enzyme is of special interest for two reasons, first because, unlike the lactic enzyme of muscle, it requires no coenzyme. In addition, it has been highly purified and shown to involve a conjugated protein containing haematin,

and is thus allied to the cytochromes. It catalyses the oxidation of L-(+)-lactic acid to pyruvic acid:

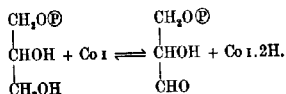


D-(-)-Lactic acid is not attacked by this enzyme, nor are any others of the β -hydroxy-acids tested. Like most dehydrogenases, the lactic enzyme of yeast can act in reverse, producing from the optically inactive pyruvic acid the L- but never the D-form of lactic acid.

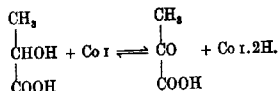
Many micro-organisms other than yeast contain similar enzymes, but in some cases at least the D-isomer of lactic acid can be formed and attacked.

Dehydrogenases requiring Co I

α -Glycerophosphate dehydrogenase (soluble). Animal tissues contain two α -glycerophosphate dehydrogenases, one of which has already been described. The soluble enzyme, unlike the insoluble, requires Co I and is specific for the D-(-)- instead of the L-(+)-form of α -glycerophosphate. Methylene blue cannot be used directly as hydrogen acceptor in this case, and the reaction catalysed is:



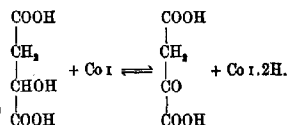
Lactic dehydrogenase of muscle has been crystallized. Like the lactic enzyme of yeast, this dehydrogenase catalyses the oxidation of L-lactate to pyruvate, again reversibly, but whereas the yeast enzyme can catalyse a direct transfer of 2H to methylene blue, that of muscle requires Co I as its immediate hydrogen acceptor:



This system plays an important part in the metabolism of muscle and other tissues, and has been studied extensively. The

equilibrium is very much in favour of the left-hand side, i.e. in favour of the hydroxy-acid, but the reaction can be forced over towards the right by adding an excess of the hydroxy-acid or, alternatively, by adding some trapping reagent, e.g. cyanide, which reacts with pyruvate to form a cyanhydrin.

Malic dehydrogenase is always found in close association with the lactic enzyme, suggesting that there may be some functional association between the two. The malic enzyme catalyses the conversion of L-malic acid into oxaloacetic acid:



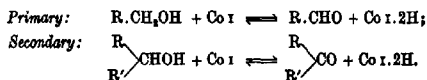
Once again the equilibrium conditions are in favour of the hydroxy-acid.

β -Hydroxybutyric dehydrogenase occurs in many animal tissues, especially in heart, kidney and liver. It catalyses the inter-conversion of L- β -hydroxybutyric and acetoacetic acids:



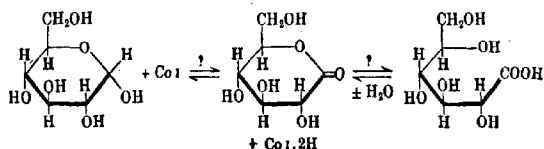
It has no action upon α -hydroxy- or α -keto-acids. The enzyme does not act upon β -hydroxypropionic acid, the next lower homologue; whether higher homologues are attacked is not known.

Alcohol dehydrogenase of yeast has been obtained in crystalline form. Like the succinic enzyme it requires the presence of its —SH groups for activity. This enzyme is very sensitive indeed to iodoacetate. A somewhat similar enzyme is present in mammalian liver. The yeast enzyme acts upon primary and secondary alcohols to yield the corresponding aldehydes and ketones:



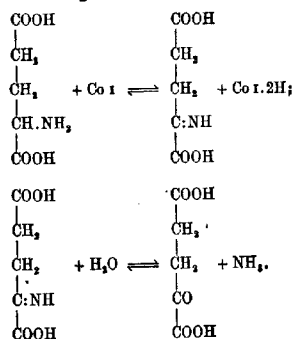
The equilibrium is in favour of the alcohols so that, in order to study the forward reactions, it is necessary to work in the presence of a fixative such as sodium bisulphite.

Glucose dehydrogenase of liver catalyses the oxidation of D-glucose to the corresponding gluconic acid. In this case it is probable that the δ -lactone is formed first of all and then reacts, possibly spontaneously, with water:



It is not known for certain whether the process is reversible. No sugar other than D-glucose is attacked by this dehydrogenase, which has the distinction of being able to use either Co I or Co II as its hydrogen acceptor.

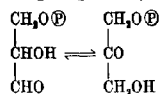
L-Glutamic dehydrogenase catalyses the conversion of L-glutamic acid to the corresponding imino-acid, a reaction that is followed by spontaneous hydrolysis of the imino-acid to yield the corresponding α -keto-acid together with ammonia:



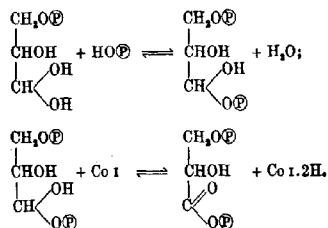
Co I and Co II are interchangeable in this system. Both stages are reversible, and α -ketoglutaric acid can be reductively aminated by ammonia in the presence of the dehydrogenase to yield L-glutamic acid. Similar enzymes are present in yeast and in plants, the yeast enzyme requiring Co II while that of plant tissues requires Co I.

This dehydrogenase appears to be absolutely specific for L-glutamic acid and has no action upon the D-isomer nor upon other L-amino-acids. Its mode of action may be compared with that of D-amino-acid oxidase (p. 101).

Triosephosphate dehydrogenase. The term 'triose phosphate' as ordinarily applied refers to an equilibrium mixture of L-3-phosphoglyceraldehyde with α -phosphodihydroxyacetone:



The so-called triosephosphate dehydrogenase is concerned with only one of these components, namely, with L-3-phosphoglyceraldehyde, and acts upon this only under certain definite conditions. Co I must be present and, as Warburg showed, inorganic phosphate also must be present. The oxidation product that accumulates corresponds not to 3-phosphoglyceraldehyde but to 1:3-diphosphoglyceraldehyde, which must accordingly be supposed to be formed as an intermediary product:



Whether the reaction of the inorganic phosphate with the starting material is catalysed by the dehydrogenase, whether it is spontaneous, or whether it is catalysed by another enzyme, is not clear. Warburg used a crystalline specimen of triosephosphate dehydrogenase in his experiments, but this does not preclude the possibility that another enzyme may have been present, on account of the tendency of proteins in general to form mixed crystals, even when still far from being chemically pure. It may be, however, that phosphoric acid can be added

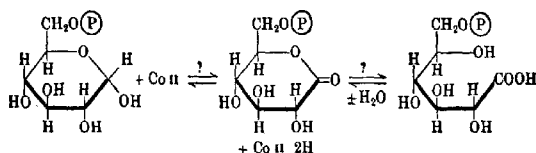
on directly, just as water can be directly added on to an aldehydic grouping.

Enzymes similar to the triosephosphate dehydrogenase of muscle are present in yeast and in plant tissues, and are probably very widely distributed indeed. The muscle enzyme is extremely sensitive to iodoacetate, from which it may be deduced that its —SH groups are required for activity. Both stages in the process which it catalyses are reversible, and the equilibrium conditions favour the formation of the acid rather than the aldehyde.

Dehydrogenases requiring Co II

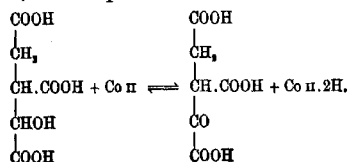
Glucose and L-glutamic dehydrogenases have already been described (p. 129).

Hexosemonophosphate dehydrogenase occurs in red blood corpuscles and in yeast. It requires Co II and catalyses the oxidation of glucose-6-monophosphate to 6-phosphogluconic acid, probably by way of the δ -lactone:



We do not know whether the process is reversible or not. This is a very specific dehydrogenase, for it has no action upon other sugar phosphates, e.g. upon fructofuranose-6-monophosphate or upon fructofuranose-1:6-diphosphate, nor does it act upon glucose itself (cf. glucose dehydrogenase).

iso-Citric dehydrogenase is widely distributed in animal tissues and its presence in other organisms is very probable. It acts upon L-iso-citrate to produce oxalosuccinic (α -keto- β -carboxy-glutaric) acid, and the process is believed to be reversible:



132 RECONSTRUCTION OF DEHYDROGENASE SYSTEMS

This is usually followed by the action of another enzyme which catalyses the β -decarboxylation of the product and gives rise to α -ketoglutaric acid.

Work on Reconstructed Dehydrogenase Systems

Most of the recent work on dehydrogenases has been carried out with the aid of what are known as reconstructed systems. It is possible, for example, to build up a system containing succinate, succinic dehydrogenase, cytochrome c, cytochrome oxidase and oxygen, and such a system can respire, i.e. can take up oxygen and oxidize an equivalent amount of succinate to fumarate. The lactic system described on p. 123 is another example of such a system. All the reactants employed are substances which occur in nature and all are obtained from living materials. It seems probable, therefore, that they represent systems which actually participate in the respiration of living cells. But there are certain important criticisms that must be noted.

The fact that a given series of operations can be demonstrated in a reconstructed system is not positive proof that it takes place under biological conditions. As Green has written: 'A sufficiently ingenious mechanic could separate the parts of a baby Austin and use them to make a perambulator or a pressure pump or a hair-dryer of sorts. If the mechanic was not particularly bright and was uninformed as to the source of these parts, he might be tempted into believing that they were in fact designed for the particular end he happened to have in view. The biochemist is presented with a similar problem in the course of his reconstructions. The materials of the cell offer unlimited possibilities of combinations and interaction, but only a few of these possibilities are realized in the cell under normal conditions. There is thus a grave element of risk in trying to reason too closely from reconstructed systems to the intact cell. The reconstruction can have no biological significance until some definite counterpart of these events is observed *in vivo*.'

It will be remembered that the oxidation and reduction of the cytochromes can, in fact, be observed within living cells, while the presence of dehydrogenases in intact cells can be

demonstrated by the Thunberg method, using a dye such as methylene blue. The discovery that the specificity of dehydrogenases is such that each can only reduce its own particular hydrogen acceptor shows that these substances, which do in fact occur in living cells of all kinds, must necessarily act as intermediates in processes of cellular oxidation. Thus the fact that the substances and the catalysts used in reconstructed systems *do* occur in intact cells, the fact that certain components *can* be observed at work within the cells themselves, and the incontestable facts of enzyme specificity—all these taken together make it seem improbable that we shall be led into gross error by the use of reconstructed systems, provided that the results are cautiously interpreted.

Intact cells, in which substances such as the cytochromes and coenzymes are present only in small concentrations, respire relatively much faster than reconstructed systems containing the same reactants at the same order of concentration. This seems at first to suggest that the two may be fundamentally different. But there are reasons for believing that whereas events in a reconstructed system take place in a more or less haphazard manner, the enzymes, coenzymes and other reactants of the intact cell are organized in such a way that each is present at the right place at the right time. We shall have other instances of this notion of intracellular organization.

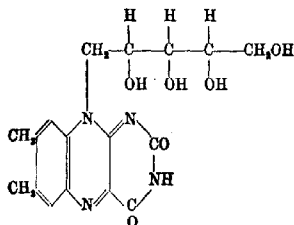
The outstanding properties of the anaerobic dehydrogenases are: their *specificity towards their substrates*, their *specificity towards their hydrogen acceptors*, and the fact that, so far as our information goes, they are all *capable of acting reversibly*. In addition, they possess the usual properties of enzymes, viz. thermolability, dependence upon pH and so on. As we have seen, *three main types can be distinguished*, the cytochrome-, Co I- and the Co II-specific types.

If now we take a dehydrogenase together with its substrate and the appropriate coenzyme, we have a system which can reduce methylene blue or cytochrome, always provided that the enzyme preparation has not been too exhaustively purified. But as purification is carried progressively further and further we find

that the system eventually fails to reduce cytochrome and methylene blue. This may be due in part to some kind of damage done to the enzymes by the methods used in their purification, but it may also mean that there must be one or more steps in the whole reaction sequence requiring catalysis by some enzyme or enzymes that are present in the crude extracts but eliminated by purification. In the case of dehydrogenases that operate through Co I and Co II there is definite evidence that additional catalysts are required to accomplish the transfer of hydrogen from the reduced coenzyme to cytochrome or to methylene blue as the case may be. The nature of these additional catalysts we shall consider in the next section.

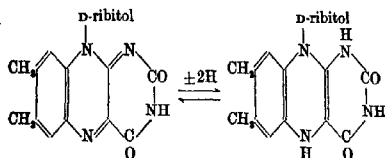
FLAVOPROTEINS

The flavoproteins are a group of conjugated proteins which are characterized by the presence in the prosthetic group of riboflavin. This is a yellow substance which exhibits a strong green fluorescence even in very dilute solutions. It is identical with the flavine of milk (lactoflavine) and with that of egg-white (ooflavine), and occurs very widely indeed among cells and tissues, its importance in which may be gauged from the fact that it is a member of the B₂ group of vitamins. It is derived from the nitrogenous base 6,7-dimethyl-iso-alloxazine and the pentahydric sugar alcohol D-ribitol, linked together in the following manner:



Certain points call for special comment. The substance can exist in the oxidized form shown above, but can be reduced by fairly powerful reducing agents such as dithionite (hydrosulphite),

Two atoms of hydrogen are taken up in the process, which may be described as follows:



Although reagents such as dithionite are necessary to effect the reduction of the oxidized form, the reduced form is autoxidizable, i.e. it can be oxidized by shaking with molecular oxygen, the oxygen being thereby reduced to hydrogen peroxide. Another point worthy of notice is the presence in this substance not of the pentose sugar, D-ribose, but of the corresponding sugar alcohol, D-ribitol, so that the name riboflavin, suggesting as it does that the molecule contains D-ribose, is a misnomer: a better name would be ribitylflavin.

Riboflavin occurs in the flavoproteins in the form of its phosphate, a substance which resembles a nucleotide in its general structure. Strictly speaking, however, it is not a nucleotide, since while it does contain a nitrogenous base it does not contain a pentose sugar. In view of its importance in cellular metabolism, in which other nucleotides also are intimately concerned, it has become common practice to refer to riboflavin phosphate as *flavine mononucleotide*. The other important nucleotides are *adenine mononucleotide* (adenylic acid) and *nicotinic amide mononucleotide*. The two latter are present in Co I and Co II, and the structures of all three mononucleotides should be compared (see pp. 295, 300).

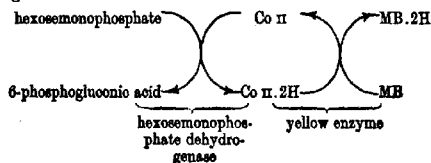
The flavoproteins fall into two classes. In the first of these the prosthetic group consists simply of flavine mononucleotide, in the second of adenineflavin dinucleotide, i.e. the dinucleotide formed by the union of flavine and adenine mononucleotides through their respective phosphate radicals. Like free riboflavin, the free mono- and dinucleotides can be reduced by dithionite, the reduced forms being autoxidizable. Similarly,

when combined in the form of flavoproteins these nucleotides can still be reduced, and when in the reduced form are usually though not invariably autoxidizable by molecular oxygen.

We have already encountered one such flavoprotein in the shape of D-amino-acid oxidase, the prosthetic group of which acts as a built-in hydrogen acceptor for pairs of hydrogen atoms which it takes over from activated molecules of D-amino-acids. That the prosthetic group is so readily reduced by activated substrate molecules when combined with the protein component of the oxidase suggests that this protein activates not only its substrates, but the prosthetic group as well, just as the dehydrogenases activate their coenzymes in addition to their respective substrates.

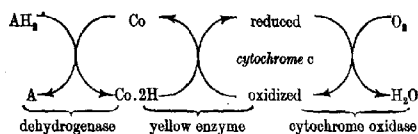
We now have to consider some other members of the flavoprotein class in which the prosthetic group again acts as a built-in hydrogen acceptor, a pair of hydrogen atoms being taken on in the same manner by the flavine nucleotide as by free riboflavine itself.

Warburg's Flavoprotein ('Yellow Enzyme'). Following the discovery that the addition of methylene blue to red blood corpuscles causes them to respire, Warburg and Christian set to work to analyse the systems involved in the respiration. They were able to show that the material oxidized is glucose-6-monophosphate, the catalyst being hexosemonophosphate dehydrogenase, requiring Co II as hydrogen acceptor. But in addition to these an additional factor was required to catalyse the transfer of hydrogen from reduced Co II to methylene blue. They subsequently isolated from yeast a flavoprotein which was found to be capable of catalysing this transfer, and gave to it the name of *Gelbferment* or yellow enzyme. The complete system may be written in the following manner:



The prosthetic group of the yellow enzyme proved to consist of flavine mononucleotide, and is apparently involved as an intermediary carrier of hydrogen between reduced Co II on the one hand and methylene blue on the other. The mode of action of this flavoprotein is thus comparable with that of D-amino-acid oxidase. The yellow enzyme is therefore an enzyme that catalyses the dehydrogenation of reduced Co II, and, as Warburg himself showed, this 'reduced-coenzyme dehydrogenase' can also catalyse the oxidation of reduced Co I in the presence of methylene blue.

The reduced form of the yellow enzyme can hand on its hydrogen to molecular oxygen, but this is a process that only takes place at an appreciable speed when the partial pressure of oxygen is relatively very high. It was formerly thought that it could transfer its hydrogen to cytochrome c also, suggesting that this flavoprotein constitutes the missing link for coenzyme-specific dehydrogenase systems in general. If this were indeed true we could describe oxidation systems involving Co I and Co II in the following general manner:



Warburg was of the opinion that this yellow enzyme must be widely distributed in nature since riboflavine, and hence by presumption flavoproteins also, are very widely distributed indeed, while living cells in general undoubtedly contain some factor which links reduced Co I and Co II with cytochrome. Up to the present, however, it has only been obtained from yeast. Attempts to detect this yellow enzyme in animal tissues have not led to evidence for its presence there, but rather to the discovery of new and different flavoproteins. Warburg himself was later reconciled to the view that the yellow enzyme is in reality an artefact derived from another flavoprotein in the course of the procedure used for its isolation.

Cytochrome Reductase. This flavoprotein, discovered comparatively recently by Haas and his co-workers, is the only member of the flavoprotein group at present known to be capable of carrying out the direct reduction of cytochrome *c*. Warburg's yellow enzyme gave some evidence of carrying out this process, but the Haas flavoprotein does so about 100,000 times faster than the yellow enzyme, and it is possible that Warburg's product was contaminated with traces of the Haas enzyme.

Cytochrome reductase resembles the original yellow enzyme in having flavine mononucleotide as its prosthetic group, but differs from it in catalysing the oxidation of reduced Co II only, reduced Co I being quite unaffected. Probably, therefore, we can look upon it as a specific dehydrogenase for reduced Co II, as well as a specific reducing enzyme for cytochrome *c*. The other essential difference between cytochrome reductase on the one hand and Warburg's enzyme on the other lies in the difference between the rates at which they reduce cytochrome *c*. As yet the reductase has only been isolated from yeast; a demonstration of its existence in animal tissues would go far towards confirming the present suspicion that it plays an important part in the oxidation of substrates by dehydrogenases that collaborate with Co II.

Diaphorase. This flavoprotein has been isolated from heart muscle. Its chief characteristics are that its prosthetic group consists of adenineflavine dinucleotide, that it catalyses the oxidation of reduced Co I specifically, that it reduces methylene blue with great rapidity, but is only very slowly oxidized by molecular oxygen. Diaphorase is thus qualified to act as a link in the reduction of methylene blue by systems involving Co I, but it does not reduce cytochrome *c*.

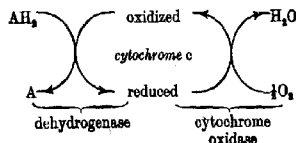
Evidence has been brought forward for the existence of a second diaphorase for the oxidation of either Co I or Co II, but there is little information about this. Thus, while we know that hydrogen can be transferred from the substrate of a dehydrogenase requiring Co II through that coenzyme and cytochrome reductase to cytochrome *c*, we do not know how a Co I-specific system transfers its hydrogen to cytochrome *c*. Presumably there must be yet another carrier that mediates between reduced

diaphorase on the one hand and oxidized cytochrome *c* on the other. It has been suggested that this further carrier may be cytochrome *b*, but present evidence is insufficient to justify any final conclusion on this point.

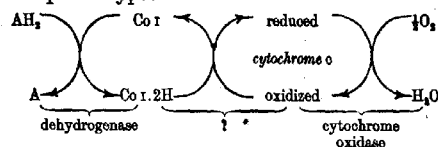
Functions of the Flavoproteins. The functions and mode of action of the flavoprotein oxidases have already been considered. Of the yellow enzyme, cytochrome reductase and diaphorase, all three behave as dehydrogenases specific for the oxidation of the reduced form of one or both of the Co I and Co II. Only one of them, however, namely, cytochrome reductase, can bring about the reduction of oxidized cytochrome *c*, and it follows therefore that our knowledge of the mechanisms of cellular oxidations is still by no means complete. We can, however, explain the reduction of methylene blue by coenzyme-specific dehydrogenase systems, through the mediation of diaphorase in cases where Co I is involved, and of cytochrome reductase where Co II is involved.

Dehydrogenases of the succinate type, requiring no coenzyme, do not appear to require the participation of a flavoprotein. Systems involving Co II require cytochrome reductase, and this flavoprotein alone suffices to establish contact between the coenzyme and cytochrome *c*. In the case of systems in which Co I participates, two possibilities seem still to be open. Either a single intermediary carrier, which cannot be identical with diaphorase, catalyses the transference of hydrogen from the reduced coenzyme to cytochrome *c*, or else diaphorase acts as a dehydrogenase for reduced Co I, passing on its hydrogen in turn to cytochrome *c* by way of some other carrier, which may possibly be identical with cytochrome *b*. Our present knowledge of the modes of action of the three known types of dehydrogenase systems can therefore be summarized in the following manner:

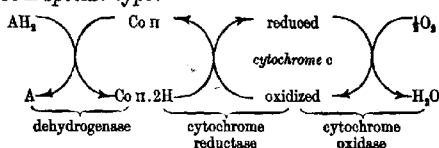
(a) Cytochrome-specific type:



(b) Co I-specific type:



(c) Co II-specific type:



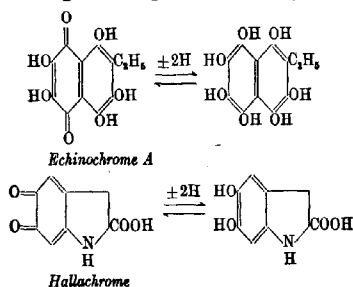
ACCESSORY CARRIERS

We have reason to believe that the bulk of the respiration of many cells is carried out by way of the cytochrome system, if only because cyanide, which inhibits cytochrome oxidase almost completely at concentrations of the order of $M/1000$, stops 80 % or more of their respiration. We have seen already that some part of the normal respiration must be attributed to the action of *oxidases*, some of which, in the presence of the appropriate substrates, are able to act as carrier systems in their own right. But most oxidases are strongly inhibited by cyanide, and it is improbable therefore that they can account for much of the cyanide-stable fraction of the total respiration.

It is known that certain cells and tissues contain carrier substances which, while reducible by the dehydrogenase systems on the one hand, are autoxidizable on the other. One such substance is *cytochrome b*, and it may be that a part of the cyanide-stable respiration is accomplished through this substance, though it is only slowly oxidized by molecular oxygen.

The occurrence of oxidases and of cytochrome *b* is very wide, and these substances may therefore be of general importance. But in special cases we know of the existence of a number of reversibly oxidizable and reducible compounds, most of which are coloured and capable of acting in much the same way as

methylene blue. If methylene blue is added to cells previously poisoned with cyanide, their respiration is largely restored, for the dye can be reduced by the dehydrogenase systems of the cells and reoxidized by molecular oxygen. As the dye is autoxidizable, its reoxidation is not inhibited by cyanide. Of the naturally occurring substances capable of acting in the same manner we may refer to *pyocyanine*, a pigment produced by certain strains of *B. pyocyaneus*, to *echinochrome*, which occurs in the perivisceral fluids and in the eggs of sea-urchins, and to *hallachrome*, which is found in the annelid worm *Halla parthenopaea*. The structures of the two last are given below, and it is interesting to notice that they are quinonoid products. A number of other substances with similar properties have been described, but it must be realized that while these compounds may well play an important part in the cells and tissues in which they occur, they cannot contribute to the cyanide-stable, nor indeed to the normal respiration, of cells in general, and do not therefore detract from the great and general importance of the cytochrome system.



In conclusion it must be pointed out that the respiration of certain cells is affected little or not at all by cyanide, and this is true of many unicellular organisms, such, for example, as *Chlorella*; the same is true of mammalian retina. In such cases it is probable either that the cytochrome system is not normally concerned in the respiration, or else that considerable amounts of other carrier substances are present. It is in fact known that cytochrome is altogether absent from some cells.

REVERSIBILITY AND COUPLING OF DEHYDROGENASE SYSTEMS

If we consider the complete system involved in the oxidation of lactic acid in animal tissues it is clear that this oxidation is accomplished through the repeated reduction and reoxidation of a chain or series of carrier substances. A single molecule of Co I, for example, might be reduced and reoxidized, say, a thousand times, and thus contribute to the oxidation of a thousand molecules of lactic acid. The oxidation and reduction of these carriers takes place with great rapidity under biological conditions, and Table 6 presents data for the 'turn-over numbers' of some oxidation catalysts, i.e. the number of times they can be reduced and reoxidized under biological conditions in 1 min. at the temperatures stated. It is precisely because their turn-over numbers are so great that very small quantities of Co I and Co II, for example, can catalyse very large amounts of chemical change. They are, in fact, catalysts, just as truly as are the enzymes with which they collaborate.

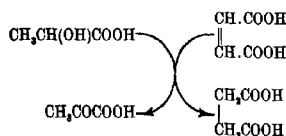
TABLE 6. TURN-OVER NUMBERS OF SOME ENZYME SYSTEMS

Enzyme	Temp. ° C.	Mol. substrate transformed per mol. enzyme per min. (approx.)
Catalase	0	2.5×10^4
Cytochrome c	38	1.4×10^3
Cytochrome reductase	25	4×10^3
Amino-acid oxidase	38	2×10^3
Polyphenol oxidase	20	7×10^4
Alcohol dehydrogenase (Co I)	20	2×10^4
Triosephosphate dehydrogenase (Co I)	20	2×10^4
Carboxylase (diphosphothiamine)	30	1×10^3

It is to be anticipated that if the supply of oxygen to the lactic acid system is cut off, the oxidation of lactic acid will cease almost immediately, since the amount of Co I available to act as hydrogen acceptor is relatively very small. This is in fact true as far as the isolated or reconstructed system is concerned. Yet many cells and tissues, including even mammalian muscle, are capable of functioning in complete absence of oxygen. It is known

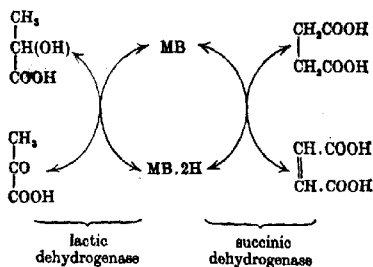
that metabolic oxidations can still go on in cells under anaerobic conditions, and in this section we have to outline the mechanisms whereby these are accomplished. It is clear that, even under anaerobic conditions, the reduced coenzymes must be reoxidized in some way since, so far as we know, they are the only biological substances that can act as hydrogen acceptors for the coenzyme-specific dehydrogenase systems.

As long ago as 1924 it was shown by Quastel and Whetham that the lactic and succinic dehydrogenase systems of bacterial cells can be coupled together in intact cells maintained under anaerobic conditions. Lactic acid is oxidized to pyruvic at the expense of the reduction of fumaric to succinic acid, thus:



At least two dehydrogenases are involved, the lactic enzyme, which catalyses the dehydrogenation of lactic acid, and the succinic enzyme, which catalyses the reduction of fumaric to succinic acid, acting in this case 'in reverse'. The mechanisms of coupled oxidation-reduction processes of this kind were much studied by Green and his co-workers, who showed that in extracts, as opposed to intact bacterial cells, the coupling can only take place in the presence of a reversibly oxidizable and reducible compound such as methylene blue. Other substances, including cytochrome *c*, and Warburg's yellow enzyme were also tested, but the only naturally occurring compound that could replace methylene blue as an intermediate hydrogen-carrier was pyocyanine, itself a reversibly oxidizable and reducible dye. In the presence of methylene blue or pyocyanine it was possible to demonstrate separately the reduction of the dye by lactate in the presence of lactic dehydrogenase, and its subsequent reoxidation by fumarate in the presence of succinic dehydrogenase. When the complete system of reactants and catalysts is taken together the conditions of equilibrium are such that a four-point

equilibrium is finally attained, and can be modified in accordance with the usual mass-law principles. Thus we may write:

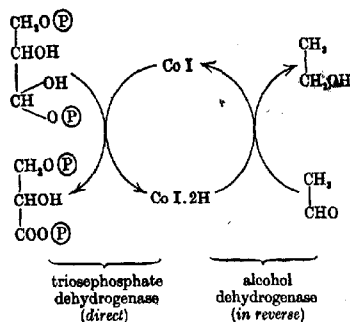


Later, working with other systems, Green and his colleagues showed that Co I also can act as an intermediary carrier between a pair of dehydrogenases, provided that both enzymes are specific for that coenzyme. A dehydrogenase that is specific for Co I could not, however, be coupled to one that normally co-operates with Co II. Many pairs of coenzyme-linked dehydrogenases have now been investigated, and the triosephosphate and lactic systems of muscle, the triosephosphate and alcohol systems of yeast, and the triosephosphate and α -glycerophosphate systems, also of yeast, are three of the many pairs that can be linked together through Co I. The L-glutamic and hexosemonophosphate systems of yeast can be similarly coupled together through Co II.

Reactions of this kind are frequently but inaccurately described as 'dismutations' and compared to the well-known Cannizzaro reaction. A true dismutation involves two molecules of one and the same substance, one of which is oxidized at the expense of the reduction of the second. Where such a true dismutation is catalysed by a single enzyme the latter is known as a *mutase*, and is a single entity as opposed to a pair of coenzyme-linked dehydrogenases.

The part played by the coenzyme in a coupled pair can be very elegantly demonstrated by taking advantage of the absorption band at 3400 A. which is shown by the reduced, but not by the oxidized, form of the coenzyme. Let us consider the coupling

between triosephosphate dehydrogenase and alcohol dehydrogenase:



If triosephosphate is taken together with the oxidized form of Co I in the presence of phosphate, no band is observable at 3400 Å. When triosephosphate dehydrogenase is added the coenzyme becomes reduced, and the progress of the reaction can be followed by measurements of the intensity of the band (Fig. 21).

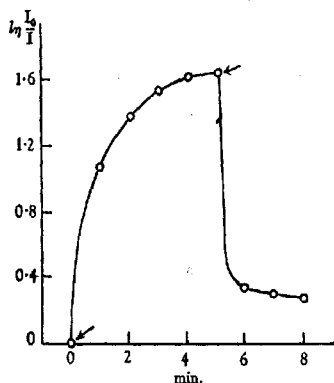


Fig. 21. Oxidation and reduction of Co I in a coupled reaction. Triosephosphate dehydrogenase added at first arrow, alcohol dehydrogenase at second; for rest of explanation see text. Ordinate: relative intensity of band at 3400 Å. Abscissa: time. (Modified after Schlenk, 1942.)

The coenzyme is not completely reduced because the reaction does not proceed to completion. The reaction mixture is now boiled to destroy the enzyme and then filtered. The intensity of the band remains unchanged, and is unaffected by the addition of acetaldehyde. If alcohol dehydrogenase is now added the second phase of the process can be observed; the coenzyme is reoxidized and the band fades.

It would be difficult to exaggerate the importance of reactions of this kind and, as we shall see later, they are a very frequent feature of metabolism under anaerobic conditions. Whether they take place when conditions are aerobic it is difficult to say with certainty. They are still *possible* under aerobic conditions, but the rate of reoxidation of reduced coenzymes by the next member in the oxidative chain is so great that these dismutation-like reactions are probably suppressed, if not abolished altogether.

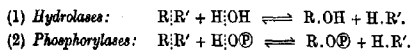
We have so far been at pains to think of these as essentially reversible systems which tend towards equilibrium. This they do in isolated systems, but when, as happens under biological conditions, one or other of the reactants undergoes some further change as fast as it is formed, the system as a whole goes in one direction only, and we shall see many examples of this kind in later chapters.

CHAPTER V

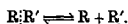
OTHER ENZYMES

GENERAL

In addition to splitting enzymes of the hydrolytic and phosphorylytic types a third group of splitting catalysts can be recognized. A typical hydrolase catalyses the hydrolytic splitting of its substrate by the introduction of the elements of water, and a typical phosphorylase a phosphorylytic splitting by the introduction of the elements of phosphoric acid:

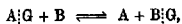


Splitting enzymes of the third type catalyse a simple, direct splitting of their substrates, without the introduction of other substances:



Enzymes of this type are best named with reference to the reverse aspect of the reactions they catalyse. Whereas hydrolytic enzymes catalyse processes of condensation when acting 'in reverse', members of the third group catalyse simple addition, and may therefore be called *adding enzymes*. They include enzymes which can catalyse the addition of water, ammonia and other substances.

Another important group of enzymes is concerned in catalysing the transference from one molecule to another of some grouping or radical such, for example, as phosphate, $-\text{NH}_2$, $-\text{CH}_3$ and so on. These may be classified as *transferring enzymes*, and the reactions they catalyse written as follows:

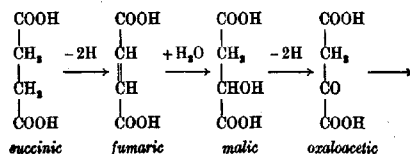


where A.G represents the donator of the radical G, and B the acceptor. The dehydrogenases and related enzymes are, of course,

a special group of transferring enzymes in which G corresponds to a pair of hydrogen atoms.

Lastly we have a group of enzymes which catalyse the inter-conversion of pairs of isomeric substances and which may therefore be called *isomerases*. It is possible in some cases at least to assign a given enzyme to more than one category, and the classification used here must be regarded as tentative and liable to revision when more has become known about the intimate details of the reactions which these enzymes catalyse.

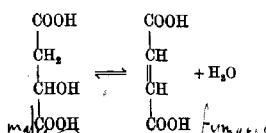
The process of digestion may be regarded as consisting of an organized series of hydrolytic reactions which results in the smooth, steady breakdown of large, complex molecules to smaller and simpler products. Hydrolytic enzymes are competent to carry the processes of digestion through from beginning to end without the aid or intervention of enzymes of other kinds. This is not true, however, of oxidizing enzymes in relation to processes of metabolic oxidation. Each dehydrogenating enzyme can catalyse only one step in the oxidation of its substrate. Succinic dehydrogenase, for example, carries the oxidation of succinate only as far as fumarate, yet if succinate is added to living cells and tissues, it is oxidized completely. Complete oxidation requires the intervention of other enzymes, one of which, fumarase, catalyses the addition of water to fumarate to yield malate, which can then be further oxidized under the influence of malic dehydrogenase:



Further modifications are required before oxaloacetate can again be dehydrogenated, and once again other enzymes come into the scheme. Thus team-work on the part of dehydrogenating enzymes requires the co-operation of enzymes of other kinds.

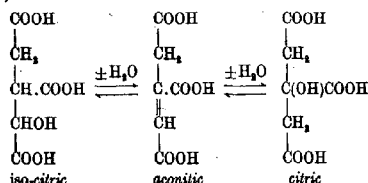
ADDING ENZYMES

Fumarase we have already mentioned: this enzyme catalyses the interconversion of fumaric and malic acids thus:

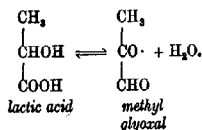


Fumarase is very widely distributed and plays a very important part in metabolic oxidations.

Aconitase also plays a part of great importance in oxidative metabolism. It might be classified either as a hydrating enzyme or as an isomerase, since it catalyses the interconversion of citric and *iso*-citric acids through the intermediate stage of aconitic acid. A water molecule can be removed from citric acid to yield aconitic acid. If the water molecule is then replaced the other way round, *iso*-citric acid is formed:

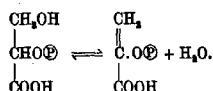


Glyoxalase, an enzyme that is very widely distributed among animal tissues, catalyses the interconversion of methyl glyoxal and lactic acid:



Its function is unknown at the present time. It requires glutathione as an activator, and the relationship appears to be quite specific, for glutathione cannot be replaced by other —SH compounds such as cysteine.

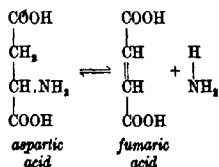
Enolase, another enzyme of great importance in oxidative metabolism and of very wide distribution, catalyses the inter-conversion of 2-phosphoglyceric and phospho-*enol*-pyruvic acids:



This enzyme requires the presence of magnesium in fairly high concentrations. It has been isolated in the form of a catalytically inert mercury compound which becomes active if the mercury is removed and replaced by magnesium, manganese or zinc. The naturally occurring enzyme is believed to be the magnesium complex on account of its extreme sensitivity towards fluoride, which forms a complex magnesium fluorophosphate in the presence of inorganic phosphate, thus preventing access of the substrate to the combining groups of the enzyme.

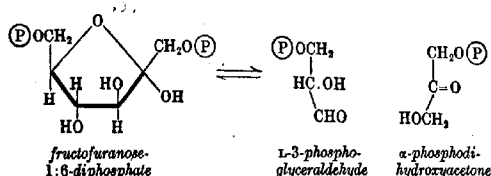
In addition to these water-adding enzymes, which may be contrasted with the hydrolases, enzymes probably exist which catalyse the addition and removal of phosphate radicals. These may be contrasted on the one hand with the phosphatases, which act hydrolytically, and with the phosphorylases, which catalyse phosphorolysis of their substrates.

Aspartase is an example of an enzyme that catalyses the addition and removal of ammonia:



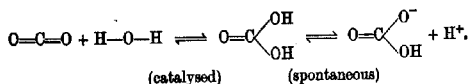
Zymohexase or *aldolase*. This important enzyme was originally discovered in yeast but is now known to be very widely distributed indeed. It catalyses the splitting of one molecule of fructofuranose-1:6-diphosphate into two molecules of 'triose

phosphate', i.e. one molecule each of L-3-phosphoglyceraldehyde and α -phosphodihydroxyacetone. The process is reversible:



Zymohexase is absolutely specific with respect to one of the products, namely, phosphodihydroxyacetone, and is group-specific towards phosphoglyceraldehyde. This can be replaced by other aldehydes, which need not necessarily be phosphorylated, so that many new compounds can be synthesized enzymatically by zymohexase, including fructofuranose-1-monophosphate for example.

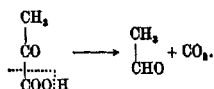
Carbonic anhydrase, an enzyme that plays an important part in the transport of respiratory carbon dioxide in the higher animals, may be regarded as a hydrating enzyme or, alternatively, as the prototype of an important group of carbon dioxide-adding enzymes. Carbonic anhydrase itself catalyses the splitting of carbonic acid to yield carbon dioxide and water and, in the reverse direction, the hydration of carbon dioxide to yield carbonic acid:



This enzyme has a wide distribution in animal tissues, and is especially abundant in erythrocytes, from which it has been prepared, highly purified, and shown to be a zinc-containing protein.

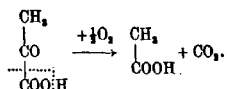
Carboxylase, originally discovered in yeast, appears to occur in some other micro-organisms and may be present in plants. It is, however, entirely absent from animal tissues. In the presence of its coenzyme, co-carboxylase, which is identical with

the diphosphate of vitamin B₁ (diphosphothiamine), carboxylase catalyses the simple decarboxylation of α -keto-acids, notably that of pyruvic acid:



There seems no reason to believe that this enzyme can act reversibly.

'*Pyruvic oxidase*' contrasts sharply with carboxylase, for it catalyses a process of decarboxylation that is essentially oxidative in nature, and is for that reason known as oxidative decarboxylation:



The so-called pyruvic oxidase is, in all probability, not a single enzyme but a somewhat complex catalytic system, as we shall see, but it resembles carboxylase in two noteworthy respects, first that it catalyses the decarboxylation of α -keto-acids, and secondly that its activity requires the participation of co-carboxylase. Like carboxylase it seems that pyruvic oxidase does not act reversibly.

Amino-acid decarboxylases, each highly specific with respect to a particular amino-acid, have recently been thoroughly investigated by Gale and his co-workers. These workers studied bacteria, many of which are able, especially in somewhat acid media, to catalyse reactions of the following general type:

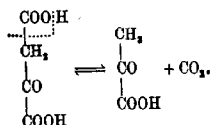


The products formed in this way include such highly toxic bodies as tyramine, histamine, putrescine and cadaverine, all of which are strongly basic and tend, therefore, to neutralize the acidity of the medium in which they are produced.

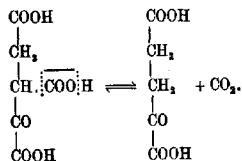
Animal tissues contain few enzymes comparable with the bacterial decarboxylases; the few that have been discovered

include enzymes that are specific for the decarboxylation of tyrosine, histidine, cysteic acid and possibly arginine. At present there is no evidence that these amino-acid decarboxylases act reversibly.

β-Carboxylase is an important and widely distributed enzyme that catalyses the reversible interconversion of pyruvic acid and oxaloacetic acid by the addition and removal of carbon dioxide as follows:



Oxalosuccinic decarboxylase similarly catalyses the interconversion of oxalosuccinic (α -keto- β -carboxyglutaric) and α -keto-glutaric acids:

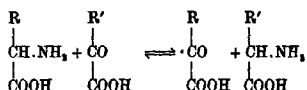


Like the other members of the carboxylase group, this enzyme has the distinction, which is shared by zymoexase, of catalysing the formation and rupture of direct carbon-to-carbon linkages.

TRANSFERRING ENZYMES

In recent years it has been discovered that there exist many catalytic systems that are capable of catalysing the transference of some group or radical from one molecule to another, over and above the hydrogen-transferring enzymes that we have already discussed. Pairs of hydrogen atoms, amino-groups, phosphate radicals, methyl- and amidine groups are among those which may thus be catalytically transferred.

Glutamic transaminase is an enzyme that occurs widely among plant and animal tissues. It was formerly believed that the $-\text{NH}_2$ group of virtually any L-amino-acid could be transferred to virtually any α -keto-acid. This process, which is reversible, is catalysed by an enzyme to which the name of aminophorase was given:

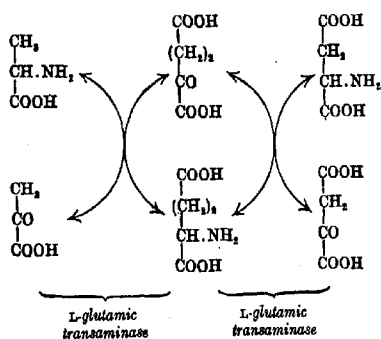


Work with more highly purified specimens of the enzyme showed that one member of the reacting pair must be either L-glutamic or α -ketoglutaric acid. L-Aspartic or oxaloacetic acids can replace these but react much more slowly. The enzyme, which is now called glutamic transaminase, is thus highly specific towards at least one of the reactants, and the general situation is to some extent comparable with that found among the dehydrogenases and their coenzymes.

It has been established that there exists a series of dehydrogenases that are collectively specific towards a common coenzyme, but each specific with respect to its own substrate. In systems of this kind the coenzyme is an obligatory reactant, acting as an obligatory hydrogen acceptor in the forward reaction and donator in the reverse process, the obligation being imposed by the specificity requirements of the activating protein, the dehydrogenase. Similarly, in the transaminase system, the enzyme requires glutamic acid as an amino-group donator, or else α -ketoglutaric acid as an amino-group acceptor. But whereas we know that there exist numerous dehydrogenases, each very specific towards some particular substrate, there is as yet no reason to suppose that there exists a group of transaminases, each specific towards one particular amino-acid or the corresponding α -keto-acid.

The part played by glutamic acid in the glutamic transaminase system is strictly analogous to that of Co I in a Co I-specific dehydrogenase system. Just as reduced Co I can pass on its pair

of hydrogen atoms to a further hydrogen acceptor, so, too, can glutamic acid pass on its amino-group to a further amino-group acceptor. The α -ketoglutaric-glutamic acid system can therefore act as an amino-group-carrying system, just as Co I and its reduced form can act as a hydrogen-carrying system. If, for example, we take alanine and add it to a purified sample of glutamic transaminase in the presence of oxaloacetic acid, no direct transference of the amino-group from alanine to oxaloacetic acid takes place. If now a catalytic amount of glutamic or α -ketoglutaric acid is also added, amino-groups are transferred from the alanine to the oxaloacetic acid so that pyruvic and aspartic acids are formed. On account of the free reversibility of the system the reaction does not go to completion but an equilibrium condition is eventually attained:

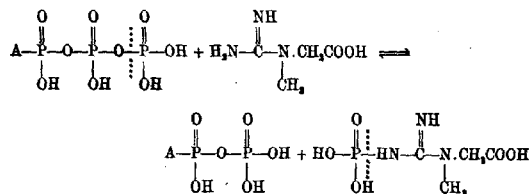


The intimate mechanisms involved in the transfer are now known and will be considered presently, since they illustrate a number of points of general importance in connexion with group-transfer reactions at large.

Aspartic transaminase. In some plants the place of glutamic transaminase is taken by a similar system in which aspartic and oxaloacetic acids replace glutamic and α -ketoglutaric acids respectively.

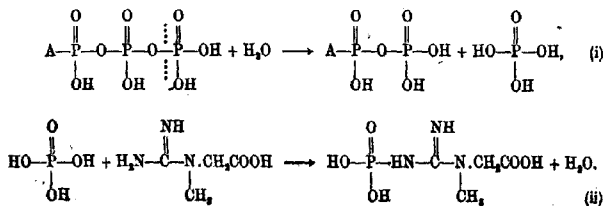
Transphosphatases

A number of enzymes are known to catalyse the transference of phosphate radicals between pairs of molecules, adenosine di- and triphosphates being employed as the carrier system. Only a few of these enzymes have so far been named, however, and they might be collectively called 'transphosphatases' or, following the suggestion of Dixon, 'phosphokinases', after hexokinase, the longest-known representative of the group. It will be convenient to consider first of all what we may call the Lohmann enzyme, since it catalyses the Lohmann reaction, i.e. the transference of a phosphate radical from adenosine triphosphate to creatine, or to adenosine diphosphate from creatine phosphate:

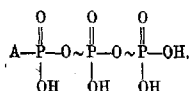


where A represents the nucleoside, adenosine.

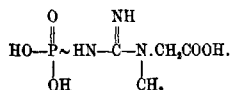
This is a particularly interesting case from the viewpoint of the mechanisms involved in group transfer. It is conceivable that the process of phosphate-group transfer might occur in two stages, thus:



Now it is known that if reaction (i) is allowed to take place, whether under the influence of the appropriate enzyme or catalysed by dilute mineral acid, the liberation of the phosphate radical is accompanied by the liberation of about 12,000 cal. of energy, in the form of heat, for each gram-molecule of phosphate set free. A second phosphate radical can similarly be set free with the evolution of another 12,000 cal. to yield adenosine monophosphate. The removal of the third phosphate radical, however, is associated with a much smaller heat production: in common with the dephosphorylation of most simple organic compounds, the energy output in this case amounts to something of the order of only 2000 cal. per g.mol. of phosphate set free. These facts are accounted for by supposing that the first two phosphate radicals of adenosine triphosphate are attached through *energy-rich phosphate bonds*, as compared with the energy-poor bond through which the third radical is attached. If we represent the energy-rich bonds by the symbol \sim , the structure of adenosine triphosphate may be expressed as follows:

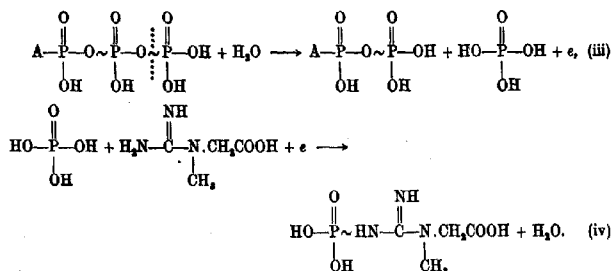


It is also known that if creatine phosphate is hydrolysed (reversal of reaction (ii)) about 10,700 cal. are set free for every gram-molecule of phosphate liberated. It follows, therefore, that creatine phosphate also contains an energy-rich bond:



It should be noticed that although the observed heat-outputs (ΔH) differ as between the hydrolysis of the terminal phosphate radical of adenosine triphosphate on the one hand, and that of creatine phosphate on the other, the changes of free energy, ΔF , are practically identical and equivalent to about 10,000 cal. in either case, so that reactions (i) and (ii) are thermodynamically equivalent.

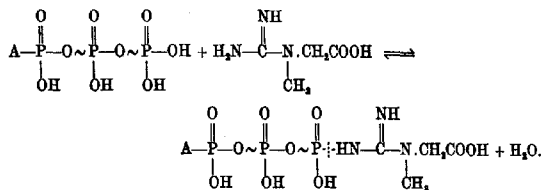
If we represent the energy associated with an energy-rich bond by e , equations (i) and (ii) may be rewritten thus:



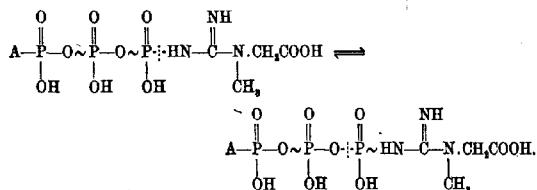
Now, when the Lohmann reaction takes place there is no significant exchange of heat, and it might therefore be suggested that the heat energy, e , of reaction (iii) is quantitatively utilized in the performance of reaction (iv). But this seems highly improbable. We should, if these equations truly represented the Lohmann reaction, be dealing with a heat engine, and heat engines seldom approach an efficiency even of 30%, let alone the 100% which is implied here. Without going further into the thermodynamic aspects of the problem, we may state that this interpretation of the process must almost certainly be erroneous. The energy represented by e cannot have a free existence since, if it did, it would in all probability be degraded as heat, the bulk of which would be unavailable. Since the liberation of e is attendant upon the liberation of a phosphate radical, with consequent rupture of an energy-rich bond, it follows that the phosphate radical cannot be actually set free.

The 'transfer' of the phosphate radical must therefore be accomplished without the removal and subsequent reattachment stages postulated in equations (iii) and (iv), and the most likely way in which this could take place is by the formation of an intermediate reaction-complex of some kind. Such a complex might be formed between the reacting materials themselves, or through the agency of a catalytic protein to which both reactants can be simultaneously attached. It is reasonable, therefore, to

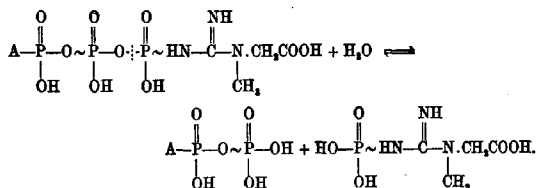
suppose that the first stage in the Lohmann reaction consists in a coupling between adenosine triphosphate and creatine, catalysed, presumably, by the Lohmann enzyme:



The complex must now undergo an intramolecular rearrangement of its bond systems in such a way that the energy distribution among the bonds is modified:



Finally, the rearranged complex may be supposed to be hydrolysed:

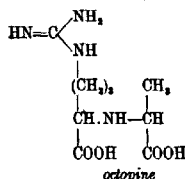


Three stages would thus appear to be involved, viz. (i) condensation of the reactants, probably catalysed by the enzyme; (ii) intramolecular rearrangement of the complex, which is perhaps an uncatalysed process involving tautomerism or resonance; and (iii) hydrolysis of the rearranged complex, which is probably also catalysed by the enzyme.

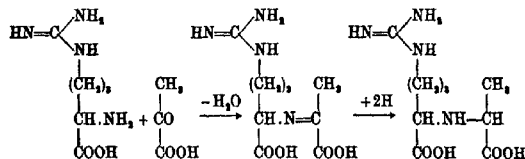
We can account in this way for the transference of the phosphate radical from one to the other of the two reactants without involving the actual detachment of the radical or the rupture of any energy-rich bond. The scheme also enables us to account for the free reversibility of the system as a whole, and for the complete efficiency of the transference of the energy.

Similar arguments apply in the cases of a number of other transfer reactions in which phosphate is involved, but it must be realized that the postulation of an intermediate complex is a matter of logical necessity rather than of experimental observation. But if we return now to the comparable process of transamination we find that there does exist experimental evidence for the formation of a tautomeric intermediate in this case.

This evidence largely comes from a somewhat unexpected source. There is present in the muscles of *Octopus*, *Pecten* and certain other molluscs a compound known as octopine, to which the following structure has been assigned:

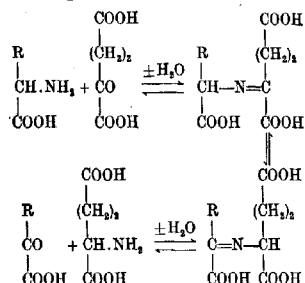


This is a post-mortem product and arises from arginine. Octopine can be chemically synthesized from arginine and pyruvic acid by simultaneous condensation and catalytic reduction, thus:



Here there is strong presumptive evidence for the existence *in vitro* of an intermediate complex formed by the condensation of an amino-acid with an α -keto-acid, precisely the type of com-

pound that would be expected as an intermediary in transamination. If we suppose that such a complex is indeed formed, we may write, for the case of the glutamic transaminase, the following reaction sequence:



Like that of phosphate, the transference of an amino-group would thus appear to involve three stages: (a) condensation of the reactants, (b) intramolecular rearrangement of the resulting complex, and (c) hydrolysis of the rearranged complex. As in the case of the Lohmann reaction we see that the radical undergoing transference has no free existence, and once more we can account in simple terms for the known free reversibility of the process. Taking the evidence all in all it does seem, therefore, that reactions of this kind probably involve the intermediate formation of some tautomeric or resonant complex which may undergo splitting in either of two ways, one leading back to the starting materials and the other to the products of the reaction.

Returning now to the phosphokinases in general, we find a group of enzymes that occur widely among living tissues and are capable of catalysing the transference of phosphate radicals from one molecule to another. In all cases known at the time of writing, adenosine triphosphate and the corresponding diphosphate are obligatory reactants. The second reactant may be any of a considerable number of substances; their identities, and the names of the corresponding enzymes, are summarized in Table 7. It is highly probable that substances other than those listed can enter into similar transphosphorylation processes, and there is

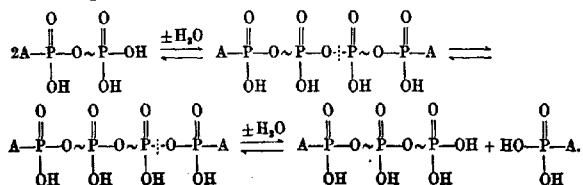
evidence that the enzymes concerned are highly specific for their substrates as well as for the carrier, which plays the part of a coenzyme. In most cases the reactions are freely reversible, but to this there are some known important exceptions.

Glucose and fructose can be phosphorylated at the expense of adenosine triphosphate under the influence of hexokinase. Phosphohexokinase catalyses a similar phosphorylation of fructofuranose-6-monophosphate. Neither of these reactions is reversible, however, presumably because neither glucose-6-phosphate nor fructofuranose-1:6-diphosphate contains an energy-rich bond. The conversion of glucose-6-phosphate to free glucose, and that of fructofuranose-1:6-diphosphate to the 6-monophosphate, follows a route different from that of their synthesis, being accomplished by hydrolysis catalysed by tissue phosphatases.

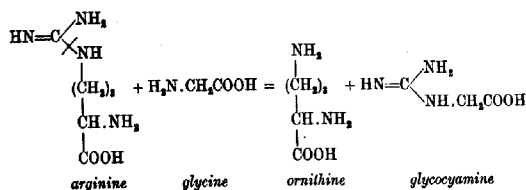
TABLE 7. PHOSPHOKINASES

Reaction	Enzyme
Creatine \rightleftharpoons creatine phosphate	'Lohmann'-enzyme
Arginine \rightleftharpoons arginine phosphate	Unnamed
Glucose \rightarrow glucose-6-phosphate	Hexokinase
Fructose \rightarrow fructofuranose-6-monophosphate	Hexokinase
Fructofuranose-6-monophosphate \rightarrow fructofuranose-1:6-diphosphate	Phosphohexokinase
3-Phosphoglyceric acid \rightleftharpoons 1:3-diphosphoglyceric acid	Unnamed
Pyruvic acid \rightleftharpoons phospho-enol-pyruvic acid	Unnamed
Adenosine monophosphate \rightleftharpoons adenosine diphosphate	Myokinase

Myokinase is a special example of the group of phosphokinases. This enzyme catalyses the transference of a phosphate radical from one molecule of adenosine diphosphate to a second, so that the products are adenosine monophosphate, on the one hand, and the corresponding triphosphate on the other. In this case it is probable that the reactions can be represented in the following manner:

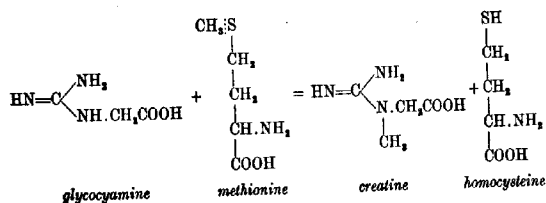


Transamidination. Enzymes exist that can catalyse the transference of the amidine group of arginine to other substances, e.g.



Little is known at present about the enzymes involved or about the mechanisms of transfer.

Transmethylation. Biological methylation is now a well-known process. It has been known for many years that the administration of pyridine to dogs is followed by the excretion of *N*-methyl pyridine in the urine. Similarly, it has been known for some time that glycocyanine undergoes biological conversion into creatine, and this change, too, involves methylation. Only recently, however, has the mechanism been discovered. The methyl groups are furnished by the amino-acid methionine, which is thereby converted into homocysteine. The following example of transmethylation makes this clear:

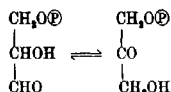


Transthiolation. In the foregoing transfer reactions, the group being transferred is exchanged for a hydrogen atom. The possibility that groups or radicals other than hydrogen may be exchanged is indicated by the transference of the —SH of homocysteine for the —OH of serine which takes place under the influence of an enzyme present in rat liver (see p. 233).

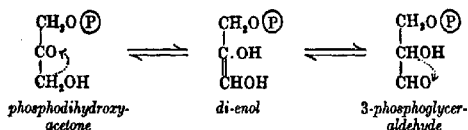
ISOMERIZING ENZYMES

There remains to be considered a group of enzymes which catalyse isomerization in their substrates. There appear to be at least two types, the first, the members of which catalyse simple isomerization, being known as isomerases.

Phosphotriose isomerase is the longest known of the group, and was formerly known simply as 'isomerase'. It catalyses the interconversion of 3-phosphoglyceraldehyde and phosphodihydroxyacetone:

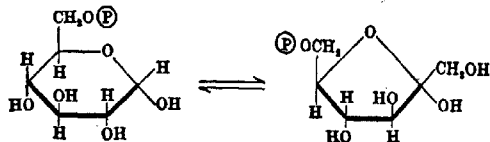


It is usually found in association with zymoheaxase, from which it has been separated and highly purified. Its mode of action is unknown, but conceivably the interconversion may take place by way of the hypothetical di-enol which is common to both substances:

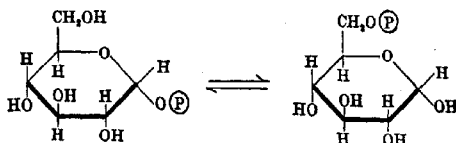


Aconitase, which we have already considered (p. 149), acts by converting citric and *iso*-citric acids into a common intermediate, aconitic acid, and it is possible that the isomerases in general act by converting the pairs of isomeric substances upon which they act into intermediate compounds which are common to both members of each pair.

Oxoisomerase catalyses the interconversion of glucose- and fructofuranose-6-monophosphates:

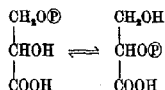


Phosphoglucomutase, which is found in association with oxo-isomerase, is a representative of another type of isomerizing enzymes, for it catalyses the interconversion of α -glucose-1-phosphate and glucose-6-monophosphate:



In this case the isomerization involves a shift in the position of the phosphate radical. Although it is not yet known how any of these enzymes act, or whether there is any fundamental difference in the mode of action of the two types, it is usual to indicate the difference by calling enzymes 'mutases' when there is some shift, such as that indicated above, as opposed to a simple intramolecular rearrangement.

Phosphoglyceromutase is another enzyme of the phosphomutase type. It catalyses the interconversion of 3-phosphoglyceric acid and the corresponding 2-phospho-derivative:



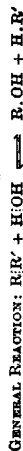
Other isomerizing enzymes undoubtedly exist. Until the mode of action of isomerizing enzymes in general becomes known it will be necessary to consider them as belonging to a class which differs from the two great classes of splitting and transferring enzymes.

CLASSIFICATION OF ENZYMES

Having now dealt with a considerable number of enzymes of different types it is convenient to attempt to classify them. The following tables present a convenient classification, but it must be realized that it is incomplete and only tentative.

I. SPLITTING ENZYMES

1. HYDROLASES



Examples

Pepsin, trypsin, chymotrypsin; katepsins I and II
Carboxy- and aminopeptidases; dipeptidase; katepsins III and IV

α - and β -Amylases; cellulase
True maltases; α - and β -glucosidases, etc.; gluco- and fructosaccharases

Pancreatic lipase; liver "esterase"; choline esterase

Bone, kidney, intestinal phosphatases

Adenosine triphosphatase; yeast pyrophosphatase

Adenase, guanase, adenylic deaminase
Glutaminase, asparaginase; urease

Class and type

PEPTIDASES:

Endopeptidases

Exopeptidases

CARBOHYDRASES:

Polysaccharases

Glycosidases

ESTERASES:

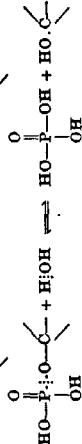
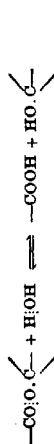
Lipases

Phosphatases

Pyrophosphatases

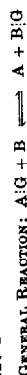
DEAMINASES

DEAMIDASES



Class and type	Reaction	Examples
DEAMIDRASES	$\text{HN}=\text{C} \begin{array}{c} \text{NH}_2 \\ \diagup \quad \diagdown \\ \text{NH} \end{array} + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{N}-\text{C}(=\text{O})-\text{NH}_2 + \text{H}_2\text{N}-\text{NH}_2$	Arginase
ANTLOPHOSPHORYLASES		
SACCHAROPHOSPHORYLASES		
NUCLEOSIDE PHOSPHORYLASES		
	<p>2. PHOSPHORYLASES</p> <p>GENERAL REACTION: $\text{R:R}' + \text{H}_2\text{O} \rightleftharpoons \text{R:O} + \text{H:R}'$</p> $\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{CH}_2-\text{O}-\text{C}-\text{O} \\ \quad \quad \\ \text{O} \quad \quad \text{O} \end{array} + \text{H}_2\text{O} \rightleftharpoons \begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{CHO} + \text{HO}-\text{C}-\text{O} \\ \quad \quad \\ \text{O} \quad \quad \text{O} \end{array}$ $\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{CH}_2-\text{N} + \text{H}_2\text{O} \rightleftharpoons \begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{CHO} + \text{HN} \\ \quad \quad \\ \text{O} \quad \quad \text{O} \end{array}$	Plant and animal (amyllo-) phosphorylases; sucrose phosphorylase Inosine phosphorylase
Substance or radical split off	<p>3. ADDING ENZYMES</p> <p>GENERAL REACTION: $\text{R:R}' \rightleftharpoons \text{R} + \text{R}'$</p> <p>Reaction</p> $2\text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2$ $\text{Malate} + \text{H}_2\text{O} + \text{fumarate} \rightleftharpoons \text{H}_2\text{O} + \text{aconitate}$ $\text{Citrate} \rightleftharpoons \text{iso-citrate} \rightleftharpoons \text{H}_2\text{O} + \text{methylyglyoxal}$ $1\text{-ketoglutarate} \rightleftharpoons \text{H}_2\text{O} + \text{methylglyoxal}$ $2\text{-Phosphoglycerate} \rightleftharpoons \text{H}_2\text{O} + \text{phospho-enol-pyruvate}$ $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$ $\text{H}_2\text{CO} \rightleftharpoons \text{CO}_2 + \text{H}_2$ $\text{CH}_3\text{COCOOH} \rightleftharpoons \text{CO}_2 + \text{CH}_3\text{CHO}$ $\text{R}_2\text{CH}(\text{NH}_2)\text{COOH} \rightleftharpoons \text{CO}_2 + \text{R}_2\text{CH}_2\text{NH}_2$ $\text{CH}_3\text{COOH} \rightleftharpoons \text{CO}_2 + \text{CH}_3\text{COCOOH}$ $\text{CO}_2\text{COOH} \rightleftharpoons \text{CO}_2 + \alpha\text{-ketoglutarate}$ $\text{Oxalacetate} \rightleftharpoons \text{CO}_2 + \text{fumarate}$ $\text{Aspartate} \rightleftharpoons \text{NH}_3 + \text{fumarate}$ <p>E.g. fructofuranose-1:6-diphosphate \rightleftharpoons phosphodi-hydroxyacetone + 3-phosphoglyceraldehyde</p>	Catalase Fumarase Aconitase Glyoxalase Enolase Carbonic anhydrase Carbonic anhydrase Carboxylase Amino-acid decarboxylases β -Carboxylase Oxalacetate decarboxylase Aspartase Zymohexase (=aldolase)
CO₂		
NH₃		
Phosphodihydroxy-acetone		

II. TRANSFERRING ENZYMES*



1. OXIDIZING AND REDUCING ENZYMES (2H transferred)

Class	Name	Group-donor	Group-acceptor
PEROXIDASES	Cytochrome peroxidase Peroxidase	Reduced cytochrome c (Unspecific)	$\rightarrow H_2O_2$ $\rightarrow H_2O_2$
	Cytochrome oxidase Monophenol oxidase Polyphenol oxidase Tyrosinase Ascorbic oxidase Uric-oxidase	Cytochromes c, c ₁ Mono- (and ? di-) phenols Di- and higher phenols Mannose and higher phenols L-Ascorbic acid Uric acid	$\rightarrow O_2$
AEROBIC OXIDASES	p-Amino-acid oxidase p-Glutamic oxidase Glycine oxidase L-Amino-acid oxidase Aldehyde oxidase (liver) Xanthine oxidase	p-Amino-acids p-Glutamic acid Glycine L-Amino-acids Aldehydes Hypoxanthine, xanthine, aldehydes Amines Diamines	$\rightarrow O_2$ [or MB ⁺]
AEROBIC DEHYDRO- GENASES	Amine oxidase Diamine oxidase 'Yellow enzyme' Diaphorase Cytochrome reductase	Reduced Co I or Co II Reduced Co I Reduced Co II	$\rightarrow O_2$ [or MB] \rightarrow (MB) \rightarrow Cytochrome c [or MB]
FLAVOPROTEINS (reduced coenzyme dehydrogenases)	Type (i) α -Glycerophosphate (insol.) Succinic Lactic (yeast)	L- α -Glycerophosphate Succinate L-Lactate	\rightleftharpoons Cytochrome c [or MB]
DEHYDROGENASES (anaerobic)			

Radical or group transferred	Class and name	Group-donor	Group-acceptor
$-\text{NH}_2$ $-\text{PO}_3\text{H}_2$ $-\text{NH}_2$ $-\text{CH}_2$ $-\text{SH}$	Type (ii) <i>α</i> -Glycerophosphate (sol.) Lactac (animal tissues) Malic Triosephosphate Alcohol (yeast) β -Hydroxybutyric Glucose (liver) L-Glutamic Type (iii) Glucose (liver) L-Glutamic Hexosemonophosphate iso-Citric	D- α -Glycerophosphate L-Lactate L-Malate L-3-Phosphoglycerolaldehyde + inorganic phosphate Primary and secondary alcohols L- β -Hydroxybutyrate D-Glucose L-Glutamate D-Glucose L-Glutamate D-Glucose-6-phosphate L-iso-Citrate	\rightleftharpoons Co I \rightleftharpoons Co II
	2. OTHER TRANSFERRING ENZYMES		
	Phosphokinases <i>(Transphosphatases):</i> Hexokinase Phosphohexokinase (Unnamed) *Lehmann enzyme* (Unnamed) Myokinase	ATP ADP	\rightarrow D-Glucose; \rightarrow D-fructose \rightleftharpoons Fructofuranose-6-phosphate \rightleftharpoons 3-Phosphoglyceric acid \rightleftharpoons enol-Pyruvate \rightleftharpoons Creatine \rightleftharpoons Arginine \rightleftharpoons ADP
	Transaminases: L-Glutamic transaminase L-Aspartic transaminase Transamidinases: (Unnamed)	L-Glutamic acid L-Aspartic acid Arginine	\rightleftharpoons α -Keto-acids \rightleftharpoons α -Keto-acids \rightarrow Glycine
	Transmethylnases: (Unnamed) Transsulfonases: (Unnamed)	Methionine Homocysteine	\rightleftharpoons Ethanolamine; \rightarrow Glycocyamine (+) Serine † MB = methylene blue.

* The arrows indicate the known directions of transfer.

III. ISOMERASES

1. SIMPLE ISOMERASES

Name	Reaction
Aconitase	Citrate (\rightleftharpoons aconitate) \rightleftharpoons iso-citrate
Phosphotriose isomerase	Phosphodihydroxyacetone \rightleftharpoons 3-phosphoglyceraldehyde
Oxoisomerase	Glucose-6-phosphate \rightleftharpoons fructofuranose-6-phosphate

2. MUTASES

Name	Reaction
Phosphomutases:	
Phosphoglyceromutase	3-Phosphoglycerate \rightleftharpoons 2-phosphoglycerate
Phosphoglucomutase	Glucose-6-phosphate \rightleftharpoons α -glucose-1-phosphate

PART II

METABOLISM

CHAPTER VI

METHODS EMPLOYED IN THE INVESTIGATION OF INTERMEDIARY METABOLISM

GENERAL PRINCIPLES

A GREAT variety of methods is available for the study of metabolic processes and it is necessary to have some idea of their applicabilities and limitations. No attempt will be made in this chapter to compile a list of all the methods that are or have been used, but rather to consider in a general way those which are most usually employed. Ideally, of course, metabolic experiments would be carried out under completely normal conditions, but this is seldom possible. The normal organism under normal conditions is a system which ingests certain materials and excretes others, and the conversion of ingesta into excreta proceeds very smoothly. In order to discover the pathways through which metabolism proceeds it is usually necessary to interrupt the normal processes in some way so as to encourage the formation and accumulation of intermediary products, or else to study the organism piece by piece. In the majority of metabolic experiments, therefore, an element of more or less serious abnormality is necessarily introduced.

In an intact, normal animal, to take a specific example, we cannot obtain much information about the metabolism of proteins by straightforward investigation of nitrogenous substances entering and leaving the organism. If proteins are fed to a mammal we find that the ingoing protein-nitrogen emerges again in

the form of urea, or in a bird in that of uric acid. Very little more can be discovered. How the nitrogen is detached from the protein, and how it is built up into urea in the one case and into uric acid in the other, we cannot discover without taking the animal more or less to pieces. If, however, we take a mammal from which the liver has been removed, it will survive for some days provided that proteins are withheld from the diet. If a protein meal is given, however, the animal quickly dies. Closer examination reveals that death is due in the main to poisoning by ammonia, and that the blood and urine alike contain ammonia, but no urea. The presence of unusually large amounts of amino-acids in the blood shows that these products of digestion are being absorbed in the usual manner; the free ammonia arises from amino-acids by deamination, chiefly in the kidneys. Deamination therefore takes place in the hepatectomized animal. But whereas ammonia set free by deamination is converted into urea in the normal animal, urea production ceases with hepatectomy. It follows, therefore, that the synthesis of urea must probably be accomplished in the liver, and further evidence regarding the mechanisms involved in that synthesis is therefore to be sought by studying the liver.

An alternative method of approach is that of feeding the intact animal with proteins or amino-acids containing heavy nitrogen (N^{15}) in place of some or all of the normal nitrogen. The isotopic form is chemically indistinguishable from ordinary nitrogen and will, we may therefore anticipate, suffer the same metabolic fate. But heavy nitrogen can be recognized and estimated by the use of suitable physical methods, and its trail through the organism can therefore be traced. Whereas in the former procedure we used abnormal animals provided with normal foods, we are now using normal animals provided with modified foods. These, in fact, represent the two fundamental methods of approach to the problems of intermediary metabolism in the intact animal. As a rule both methods are used. But even when the broad, main lines of metabolism have been traced out, there still remains the task of analysing them into their separate stages and steps so that, starting with a whole, intact organism,

we find ourselves studying organs, tissues, tissue extracts, groups of enzymes and even single, highly purified enzymes, as the work of analysis proceeds.

In general it must be pointed out that all the methods at present available for metabolic studies, whether in whole cells or in whole organisms, are liable to lead to erroneous conclusions on account of abnormalities introduced by the experimental conditions. The fact that such-and-such a reaction can be demonstrated in a given animal preparation or tissue extract is no guarantee that the reaction is one which normally takes place. Evidence from no one source should be regarded as absolutely conclusive, no matter how convincing it may appear: in every case evidence obtained by one method should be checked against evidence obtained by another with different inherent limitations. The fact that individual workers tend to develop and adhere to one particular technique is much to be regretted, even though the specialization of biochemical methods is rapidly becoming so intense as to make it virtually impossible for any one worker to master more than one or two techniques.

STUDIES ON NORMAL ORGANISMS

The chief method in which normal, intact organisms are used consists essentially in the administration by feeding, injection or otherwise of the material the metabolism of which is to be investigated, followed by the examination of the tissues and excreta for possible intermediate products.

If we assume that a given compound *A* undergoes conversion through a series of intermediates, *B*, *C*, *D*, etc., to yield in the end a product *X*, it will in general be possible to detect, isolate and identify *X* among the excreta. But even this seemingly simple procedure is beset with pitfalls. The substance *X*, we will suppose, is found in the urine. Unless the urine is analysed very soon after it has been passed, or else is treated with toluene or some other preservative, there is every prospect of heavy bacterial contamination which may transform *X* into some other substance or substances. The same danger arises much more

acutely in the case of faecal analysis for here, even before the faeces are voided at all, they will already have been incubated for some hours in the presence of a massive bacterial population, and under conditions which are about optimal for bacterial growth. To attain complete faecal sterility is virtually impossible and, in consequence, faecal analysis plays a relatively small part in most metabolic studies. This particular difficulty has been overcome in some cases by opening the upper regions of the intestine to the surface by surgical operation, but the animal cannot then be regarded either as intact or as strictly normal.

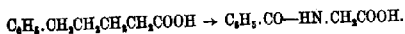
The possibility of bacterial involvement is also of major importance in work on herbivorous animals, most of which maintain in their alimentary tract great populations of symbiotic micro-organisms upon which they rely for the digestion of much of their food. Any substance fed to such an animal will have to run the gamut of these symbionts before it reaches the blood stream of their host, and may be more or less extensively modified in the process. In experiments upon herbivorous animals, such as the rabbit, it is therefore necessary to inject the material to be studied, whether subcutaneously, intravenously or otherwise. For direct-feeding experiments carnivorous animals such as dogs and cats are usually preferred, since the inconvenience of injection can thus be avoided. And this fact alone raises further complications, for herbivorous animals and carnivores may differ considerably in their metabolism. It is necessary therefore to be on one's guard against the temptation to argue from a carnivore to a herbivore, and from one species to another.

We have so far considered only the end-products of metabolism. Several methods may be used to discover the nature and identity of the intermediates *B*, *C*, *D*, etc. The usual procedure is to administer massive doses of *A*. In a reaction sequence $A \rightarrow B \rightarrow C \rightarrow D \rightarrow$ and so on, the rate of the process as a whole will be limited by that of the slowest reaction in the chain, say $D \rightarrow E$. If the system is overloaded by giving massive doses of *A*, *D* will tend to accumulate and may therefore appear in the excreta, blood or tissues, in any or all of which it may be sought. But the administration of massive quantities of *A* may have conse-

quences that we do not anticipate. It is possible that *A* may be transformed along more lines than one, so that, when the concentration is high, abnormal side-products begin to accumulate. These may be discovered and mistaken for normal intermediates. Again, if *D*, which in the ordinary way is converted into *E* as fast as it is formed, attains any appreciable concentration in the tissues, it, too, may be converted into abnormal side-products which, once more, may be mistaken for normal intermediates. It is usual, therefore, to administer suspected intermediates in fresh feeding or injection experiments and see whether they yield the same products as normally arise from *A* itself.

Another possibility is that *A* may normally undergo conversion into several products, *B*₁, *B*₂, *B*₃ and so on, one of which, say *B*₂, is excreted without further change. Since the only apparent intermediary we shall detect in such a case is *B*₂, we may be misled into believing that the whole of *A* is normally transformed into *B*₂. It is necessary that these various possibilities should be kept in mind in the interpretation of results obtained in feeding or injection experiments: provided they are realized and that due allowance is made for them, valuable information can usually be obtained. Experiments of this kind have done yeoman service in the past and will doubtless continue to do so in the future.

A further method in which intact, normal animals are used involves the chemical alteration of the substance *A* in such a manner that it and its products can more easily be detected and recognized. Thus Knoop, in his classical experiments on the metabolism of fatty acids, introduced a phenyl radical into the terminal position of the fatty chain and was able then to find aromatic derivatives in the urine of animals to which these ω -phenylated fatty acids had been administered; ω -phenylvaleric acid, for example, gave rise to hippuric acid when given to dogs:



It was already known that the administration of benzoic acid to dogs gives rise to the appearance of hippuric acid in the urine,

and Knoop was therefore able to conclude that phenyl valeric acid is converted into benzoic acid by the animal's tissues.

This method is open to a number of serious objections. First, we cannot assume that if we modify the starting material we shall not alter its fate in the organism, nor, secondly, can we assume that by feeding abnormal material we shall not induce some completely new series of reactions which, in the ordinary way, play no important part in metabolism.

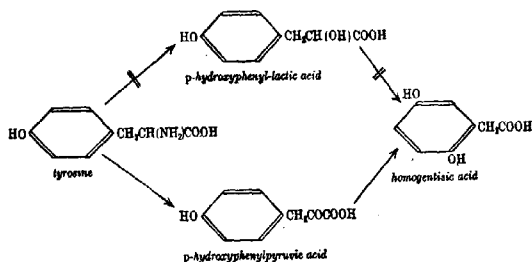
Valuable information has nevertheless been gained in the past from work of this kind, and the method has its present-day counterpart in the use of isotopes, such as heavy hydrogen, heavy nitrogen, radioactive carbon and so on, as 'tracers'. These isotopes are not chemically distinguishable from the normal forms, and it may reasonably be supposed therefore that their metabolism will follow normal lines. The isotope method is used extensively at the present time.

STUDIES ON ABNORMAL ORGANISMS

Organisms that are intact but suffering from some pathological derangement of metabolism offer valuable experimental material for some purposes. Certain very special metabolic abnormalities occur spontaneously, though rarely for the most part. Albinos, for example, are devoid of the enzyme tyrosinase, and may be used in studies of the metabolism of the aromatic amino-acids. The metabolism of tyrosine goes astray in a number of other curious genetic freaks, notably in alcaptonuria, a disorder in which the urine becomes dark brown or black when allowed to stand exposed to the air. The blackening is due to the presence of a diphenol, homogentisic acid, which arises from the aromatic amino-acids. When the urine is allowed to stand, bacterial invasion takes place and ammonia is formed from urea by the invading organisms. Like many other diphenols, homogentisic acid undergoes spontaneous oxidation in alkaline solution to yield dark-coloured products.

Cases of alcaptonuria were studied in an attempt to decide whether amino-acids undergo oxidative or hydrolytic deamina-

tion. Homogentisic acid is no longer excreted if aromatic amino-acids are excluded from the diet. It is reasonable therefore to suppose that any substance which lies on the route between tyrosine and homogentisic acid will, if administered to an alcaptonuric deprived of aromatic amino-acids, give rise to a renewed excretion of homogentisic acid. Now if tyrosine were hydrolytically deaminated the deamination product would be *p*-hydroxyphenyl-lactic: if oxidatively, the first product would be *p*-hydroxyphenylpyruvic acid. Both these substances were accordingly prepared and separately administered to human patients suffering from alcaptonuria. It was then found that whereas *p*-hydroxyphenylpyruvic acid was almost quantitatively converted into homogentisic acid, none was formed from the corresponding hydroxy-acid. The relationships of these substances are as follows:



It was concluded that the deamination of tyrosine, and by inference that of other amino-acids, is an oxidative rather than a hydrolytic process.

The danger-points in this argument are, first, the supposition that because *p*-hydroxyphenylpyruvic acid yields homogentisic acid it necessarily lies on the pathway tyrosine \rightarrow homogentisic acid: it might equally well form homogentisic acid by some independent and possibly abnormal route. Secondly, even if we discount the first objection and take it as established that tyrosine is in fact deaminated with production of the corresponding keto-acid, it is exceedingly dangerous to assume that amino-acids

other than tyrosine also undergo oxidative deamination, if only because, in alcaptonuria, the metabolism of tyrosine itself is seriously deranged.

Particularly important among the pathological conditions of which advantage has been taken is the state of diabetes. Spontaneous diabetes, diabetes induced by surgical removal of the pancreas or by injection of the diabetogenic hormone of the anterior pituitary, and the pseudo-diabetes induced by injection of the drug phlorrhizin, have all been put to service, especially in studies of the metabolism of fats and carbohydrates. These animal preparations have two important features in common. First, carbohydrate metabolism is profoundly deranged and glucose, instead of being stored in the liver in the form of glycogen, is eliminated in the urine. Secondly, there is a large-scale excretion of the so-called acetone or ketone bodies. These compounds, acetoacetic and β -hydroxybutyric acids, together with acetone, are formed from fatty sources. If a diabetic or phlorrhizinized animal is maintained on a constant diet, a steady rate of excretion of glucose and acetone bodies can be established. If now substances such as alanine, lactic acid, glycerol and the like are administered, an increased output of glucose ensues, indicating that these substances give rise to or replace carbohydrates in the organism. Other compounds, such as butyric and acetic acids, together with the amino-acid leucine, increase the excretion of acetone bodies. The diabetic or phlorrhizinized animal is thus useful as a device which allows us to detect the formation of carbohydrate and fatty materials from substances of other kinds.

Particularly important among the surgical preparations is the hepatectomized animal. Total removal of the liver is a difficult operation, and the subjects do not survive for more than a few days. An alternative procedure is to establish an Eck's fistula, i.e. to by-pass the liver by leading the portal blood directly into the inferior vena cava. The liver plays a leading part in many metabolic processes, and in its absence these are thrown out of gear or even stopped altogether. Intermediary products tend to pile up and commonly appear in the urine. Mention has already

been made of one such case: ammonia produced by the deamination of amino-acids is normally converted into urea by mammalian liver, and into uric acid by the liver of birds, but these synthetic operations cease with removal of the liver or establishment of an Eck's fistula, amino-acids and ammonia accumulating instead. This tells us that urea and uric acid are formed from ammonia and that their synthesis takes place in the liver, but gives no indication of the mode of synthesis. It does, however, serve to show what particular organ we must study in order to elucidate the rest of the story.

The hepatectomized animal is of particular value on account of the central metabolic role of the liver, but pancreatectomized, adrenalectomized, hypophysectomized, thyroidectomized and other preparations have been much employed, especially in attempts to discover the parts played by hormones in the regulation and control of metabolic processes. In all such cases the preparation is abnormal in certain known respects, but it is necessary to realize that processes other than those which we know to be deranged may also be thrown out of gear. Confirmation of results obtained by one method should therefore always be sought, and usually is sought, with the aid of other methods and different preparations.

STUDIES ON PERFUSED ORGANS

It is often possible to study the metabolic activities of a particular organ by providing it artificially with an independent circulation. The organ to be perfused may either be left *in situ* in the animal, or may be removed and kept under conditions that approximate as closely as possible to those which it enjoys under normal physiological conditions. The circulating medium may be the animal's own blood, or blood drawn from another individual of the same species; alternatively, it is possible to use certain physiological salines which we shall discuss presently. The necessary head of pressure may be obtained by means of mechanically operated pumps arranged to imitate the action of the heart, and many types of 'artificial lungs' have been devised for oxygenation of the medium.

The method of artificial perfusion is open to a number of serious objections. It takes time to establish the artificial circulation, while the animal must of course be anaesthetized during the operation. This means that, for the first few minutes, the organ is liable to be influenced by temporary deprivation of oxygen and by the anaesthetic. The choice of anaesthetic has therefore to receive careful consideration. How much damage may be done by the temporary shortage of oxygen is not easy to ascertain but, given speed and skill, damage from this cause can be minimized. If blood is used for the perfusion it is necessary to add an anti-coagulant such as heparin, and the possible action of this upon the organ has to be reckoned with. Care must be taken to ensure that the perfusion medium is kept at the right pressure, temperature, pH and so on, and that it shall be kept well oxygenated, but all these are largely technical matters which can be dealt with, given experience and skill on the part of the operator.

There are, however, other objections that cannot so easily be countered. So long as the organ enjoys its normal blood supply it is exposed to nervous and hormonal influences which cannot be exactly reproduced outside the animal. Thus the liver, a favourite object of study by the perfusion method, plays a great part in the metabolism of carbohydrates, and this, as we know, is profoundly affected by a number of hormones, notably by insulin, adrenaline and the diabetogenic hormone. When the liver is removed from the body the influence of these is withdrawn, and this may be expected to result in abnormal metabolic behaviour. Thus, from the moment at which the normal circulation is replaced by the experimental perfusion system, the organ is exposed to conditions which are abnormal, and probably become progressively more abnormal as time goes on. It is always difficult to be certain that the whole of the organ is actually being perfused, so that parts may be moribund or dead long before the whole. Nevertheless, it does seem that, for the first hour or two, a skilfully manipulated preparation behaves in a manner which approximates fairly closely to normal, and results obtained with such preparations commonly find confirmation by other techniques.

The general procedure, following the successful establishment of the artificial circulation, consists in adding substances of which the metabolism is to be studied to the circulating medium, samples of which are withdrawn from time to time for analysis. Perfusion experiments may be done with liver, muscle, heart, kidney and so on. Large animals are usually preferred since large size facilitates the operative procedure, though it makes considerable demands upon laboratory accommodation at the same time. The recent introduction by Trowell of a technique for the perfusion of rat liver may do much to restore the method of liver perfusion to some of its former eminence.

As well-known examples of the successful employment of this method we may refer to the classical observation that isolated, perfused dog liver synthesizes urea from added ammonia, goose liver producing uric acid by contrast; and, in addition, to the work of Embden, Friedmann and others on the metabolic fate of the fatty acids.

THE USE OF PHYSIOLOGICAL SALINES

Perhaps the most important single contribution ever made to physiology and biochemistry was the discovery in the early 1880's by Sidney Ringer that simple solutions of the chlorides of sodium, potassium and calcium can maintain the action of the perfused hearts of frogs and tortoises. Subsequent work has shown that with the aid of slightly more complex media the heart-beat of warm-blooded animals also can be maintained for many hours or even days. There is reason to think that, of all the multifarious constituents of mammalian blood, many are specialized features of secondary importance, the ionic constituents alone being absolutely fundamental and essential. Given a supply of well-oxygenated physiological saline at the appropriate temperature, pH and osmotic pressure, it seems that the fundamental physiological requirements even of mammalian tissues can be fulfilled.

Numerous salines have been introduced for specific purposes. They can be used, for example, to replace blood in perfusion experiments, and have been used clinically on a large scale in the past to make up the blood volume in cases of severe haemorrhage.

Solutions containing the chlorides of sodium, potassium, calcium and magnesium, together with small amounts of phosphate, are suitable for many purposes, and are best buffered with bicarbonate and carbon dioxide. Glucose is often added to provide 'food' for the tissues. The ionic composition of one such saline is given, side by side with that of the mammalian blood it is designed to imitate, in Table 8; this particular medium has been used extensively by Krebs and others in work involving the use of tissue slices.

TABLE 8. COMPOSITION OF MAMMALIAN BLOOD SERUM
AND KREBS'S PHYSIOLOGICAL SALINE

	Mammalian serum (averages)	Physiological saline
Na ⁺	a. 320	327
K ⁺	22	23
Ca ⁺⁺	10	10
Mg ⁺⁺	2.5	2.9
Cl	370	454
PO ₄ ⁻	10	11
SO ₄ ⁻	11	11.4
HCO ₃ ⁻	54 vol. %	54 vol. %
CO ₂ (at 38° C.)	2.5 vol. %	2.5 vol. %
pH	7.4	7.4

All concentrations are in mg. per 100 ml., excepting bicarbonate and carbon dioxide, which are expressed as ml. CO₂ per 100 ml. *Glucose* (0.2%) is also added before use.

USE OF TISSUE SLICES

In recent years the somewhat messy method of perfusion, making as it does considerable demands upon the surgical skill of the experimenter, has been largely replaced by the use of tissues in the form of thin slices. Provided certain conditions are fulfilled, these slices will survive for some hours, apparently in a manner that approximates closely to the physiological, and are simple to prepare and manipulate. The size of the average cell is such that, although many cells are inevitably damaged when the tissue is sliced, the proportion of damaged to undamaged is very small, while the debris of those that are damaged can be removed fairly completely by washing. Provided the organ to be used is removed, sliced and washed rapidly, we can obtain small frag-

ments of virtually normal tissue. Their removal from the normal blood supply of course implies that they are removed also from the influence of the animal as a whole, just as is the case in perfusion experiments, but whereas an unknown, and often a considerable, proportion of the cells in a perfused organ is probably in a poor, if not actually moribund, condition, washed tissue slices contain relatively few cells that are appreciably injured.

Certain conditions must be fulfilled in the preparation and use of these tissue slices. It is usually convenient to have fragments one or two centimetres square. Their thickness must be such that the cells in the middle of the slices, which can acquire oxygen only by inward diffusion from the medium, do not suffer from lack of oxygen. Usually, therefore, the medium is kept in equilibrium with an atmosphere containing 2.5-5% of carbon dioxide for buffering purposes, and 97.5-95% oxygen. If such a gas mixture is used the slices must not be more than 0.3 mm. thick. Satisfactory slices can fairly easily be cut free-hand with a sharp razor.

The usual procedure is as follows. Suitable vessels are filled with the appropriate saline, through which the gas mixture is bubbled, the whole being gently shaken in a thermostatic bath at body temperature. Other samples of the medium are prepared for washing the slices. The animal is killed, by a blow on the neck for example, and the organ required is rapidly removed and placed on clean blotting-paper moistened with warm saline. Slices are rapidly cut and placed at once in the warm, oxygenated saline until enough have been accumulated. They are next washed two or three times and then transferred to the main vessels. The substances to be studied are added and the whole apparatus is shaken for a suitable period, during or after which samples of the medium are withdrawn, deproteinized if necessary, and analysed.

The method is open to several of the criticisms that apply to the perfusion technique, though others are obviated. No anaesthetic is necessary, and a small animal such as a rat or a guinea-pig will supply enough material for a number of experiments. It is possible that the cells may behave abnormally as a result of

their exposure to the high partial pressures of oxygen required to ensure adequate oxygenation of the deeper layers of cells. The method has, however, found wide favour. On account of the small size of the tissue fragments, the method is very suitable for the application of manometric methods which, as is well known, can be used for a very wide range of measurements and estimations.

USE OF BREIS AND EXTRACTS

The analysis of a complex series of metabolic events into its component reactions usually provides evidence for the participation of a number of enzymes and accessory catalysts, and for a complete analysis the identification and the discovery of the function of each of these is required. To obtain this information it is necessary to separate the enzymes one from another, to destroy some enzymes and preserve others, or in some other way so to disrupt the cellular organization that intermediate products can be discovered. Sometimes this can be done by the use of specific inhibitors known to inactivate particular enzymes; in other cases 'trapping' reagents can be employed to fix particular intermediates. More usually it is necessary to extract the enzymes from the tissue, though it sometimes suffices to mince or grind the tissue. The resulting minces and 'breis' contain all the enzymes of the original material, but the spatial relationships between them have been destroyed by the disruption of the cellular architecture. Often it is necessary to go further. Many enzymes can be extracted with water or saline, freed from the general cell debris by filtration or centrifugation, and later purified. Others are insoluble, and can best be studied in the form of exhaustively washed minces or breis. Of the soluble enzymes some tolerate precipitation with acetone at 0° C. and enzymes of this kind can be extracted with aqueous media, precipitated by means of acetone, and then extracted again with water or saline from the resulting 'acetone powders'.

A fine example of the usefulness of whole extracts is found in the case of yeast juice, which is prepared by macerating the cells with sand and squeezing the mass in a hydraulic press.

Many of the enzymes extracted in this way require coenzymes, which can be removed by dialysis, and much of our present knowledge of fermentation has been gained by the use of dialysed yeast juice.

In the end it is usually necessary to have recourse to purified enzymes. Finely divided tissue is allowed to stand with water or with isotonic potassium chloride, for example. After centrifugation of the extract it is possible to purify many enzymes by fractional precipitation, fractional adsorption, and other more specialized procedures so that highly concentrated preparations are obtained. By further rigorous purification, the details of which vary according to the nature of the enzyme, crystalline preparations are obtainable in a number of cases. It is as well to remember, however, that proteins in general—and enzymes are no exception to the rule—are very prone to the formation of mixed crystals while still far from being chemically pure.

Detailed knowledge of the processes catalysed by a single enzyme often helps us to analyse into several stages a process which seems at first sight to be a single metabolic operation, and information gained by the study of 'built-up' or reconstructed systems comprising several enzymes and their appropriate accessory catalysts, may give valuable indications of the manner in which the individual stages are organized in the metabolic whole. We shall come across numerous examples of this kind, but for the moment the reader may be reminded of the use of reconstructed systems in the study of the dehydrogenases, and Green's warning apropos of these reconstructions may also be recalled (p. 132).

CHAPTER VII

FOOD, DIGESTION AND ABSORPTION

FOOD

LIVING organisms can be broadly divided into two groups. Some, like the green plants, only require to be provided with simple inorganic materials from which, with the aid of energy drawn from the external world, they can accomplish the synthesis of everything required for their life, growth and reproduction. Others, like the animals, can only live and reproduce if provided with complex, energy-rich, organic materials, collectively designated as food. These two groups of living organisms are known as *autotrophes* and *heterotrophes* respectively.

Predominant among autotrophic organisms are the *green plants*, which are able to fixate and utilize the energy of solar radiation. This is brought to bear, in a manner at present unknown, upon the synthesis of complex energy-rich materials; and, as raw materials for the synthesis, carbon dioxide, water, salts, and simple sources of nitrogen such, for instance, as ammonia or nitrates are necessary. The key substance in photosynthesis, chlorophyll, finds counterparts in the specialized bacterial pigments upon which the *photosynthetic bacteria* rely for a comparable fixation of solar energy. In the remaining group of autotrophic organisms, the *chemosynthetic bacteria*, energy is not obtained from the sun, but by harnessing the chemical energy of some inorganic process such as the oxidation of ammonia to nitrite or nitrate, the oxidation of hydrogen sulphide to elemental sulphur, or that of ferrous compounds to the ferric state. The autotrophes are in every case competent to synthesize all the structural, catalytic and storage materials they need for growth, maintenance and reproduction: everything their life requires they can produce from the simplest of starting materials, the necessary energy being collected from the external world.

Heterotrophic organisms stand in sharp contrast to the autotrophes, for not even the most versatile of heterotrophic forms can live except by exploiting the industry and synthetic ingenuity of other organisms. Only by fermenting, oxidizing, or in some other way degrading complex organic material can the heterotrophes obtain the energy required to maintain themselves. It may therefore be said that all heterotrophes require 'food', in the sense of oxidizable or fermentable material, by the breakdown of which energy can be released and harnessed for locomotion, chemical synthesis, and other energy-consuming processes.

Many heterotrophic forms of life such, for example, as the *free-living bacteria* and *yeasts*, can live and reproduce in very simple media. Apart from water, salts and some simple source of nitrogen, they need only to be provided with some fairly simple organic compound such, say, as lactate. Given these substances the free-living bacteria can accomplish *de novo* the synthesis of everything their life requires. But many micro-organisms are more exacting. The presence of certain particular compounds in the habitual environment of a given species can lead to the loss within that species of the ability to synthesize the substances in question. Thus many milk-souring bacteria, such as are cultivated for the manufacture of cheese, cannot live or multiply except in media containing riboflavine. Free-living organisms are able to synthesize this important substance for themselves, but the cultivated milk-sourers have lived for so many generations in milk, which is a fairly rich source of riboflavine, that their ability to synthesize it has been lost for lack of employment. For these organisms, riboflavine has become an indispensable accessory food factor; in other words, a vitamin.

The nutritional requirements of many micro-organisms have been carefully investigated in recent years, and there is now abundant evidence that some degree of synthetic disability is a common feature among them. Yeasts which have been carefully nursed and pampered in vineyards and breweries require the provision of a number of the factors that free-living forms can make for themselves, while among bacteria and Protozoa many

forms, including numerous highly pathogenic strains and species, have been found to have nutritional requirements that are very exacting indeed. Loss of synthetic ability seems to be a step-wise process, for certain bacteria, given β -alanine or nicotinic amide, can synthesize pantothenic acid and Co I respectively, but in other cases it is necessary that these more complicated substances should be given intact; even the ability to join together the constitutional fragments has been lost. This loss of synthetic ability has gone even further in certain micro-organisms than it has in animals, for some of them are unable even to synthesize haematin.

By contrast with green plants, or even with free-living micro-organisms, *animals* are very exacting creatures indeed. In addition to water, salts, and 'food' in the sense of energy-yielding organic substances, animals of every kind need to be provided with certain amino-acids, and with a number of other indispensable accessory food factors collectively known as vitamins. These include thiamine, riboflavine and nicotinic amide, all three of which are constituents of essential coenzymes. This implies the inability of animals to synthesize many of the tissue constituents and catalysts which they require.

These general notions help greatly in the interpretation of the food relationships that exist between living organisms of different kinds. In the words of Charles Elton, 'Animals are not always struggling for existence. They spend most of their time doing nothing in particular. But when they do begin, they spend the greater part of their lives eating. The primary driving force of all animals is the necessity of finding the right kind of food and enough of it.' The 'right kind of food' is largely determined, of course, by the animal's ability to capture and kill other organisms, but on the chemical side we can say that the 'right kind of food' is that which provides the eater with the energy requirements of its kind and, at the same time, with whatever special materials are essential to the species in consequence of its synthetic disabilities.

In any natural animal and plant community we can trace out what are known as food chains. A food chain typically begins

with green plants, which are exploited by herbivorous animals and these, in their turn, by carnivores. These become the prey of larger and more powerful carnivores and so on until, in the end, we arrive at an animal so large and powerful that it has virtually no enemies except, perhaps, that ubiquitous animal, man. Always in these food chains the starting-point is with autotrophic organisms. Herbivorous animals rely at first hand, and carnivores at second or third hand, upon the autotrophes for supplies of the numerous essential substances which they require, as well as for a sufficiency of complex, energy-yielding organic foodstuffs. Gathered together in the first instance by herbivorous beasts, these essential materials are passed stage by stage along the food chains.

The same general ideas are also valuable in the interpretation of food relationships of other kinds. *Parasitism*, for example, presents many problems which a thorough knowledge of the nutritional requirements of parasitic organisms may go far towards solving. We have already seen that the requirements of a given organism are liable to be influenced by the availability of particular substances in the environment to which the organism is accustomed, quite apart from the general physico-chemical properties of the habitat. Many micro-organisms are now confined to particular habitats and have, in fact, become essentially parasitic upon those habitats because they have lost the ability to synthesize certain substances; they can therefore only survive in environments in which those substances are to be found. Very possibly the same will prove to be true of other parasites, such, for instance, as the tape-worms and round-worms that are such a common feature of the intestinal fauna of animals of every kind.

Another important type of nutritional association is *symbiosis*. A cow may harbour large numbers of parasitic worms, in addition to the multitude of symbiotic micro-organisms inhabiting its rumen, but the relationships between the cow and the worms, on the one hand, and between the cow and its symbionts on the other, are very different. The cow acts virtually as a food-collecting machine for both groups of organisms, but gets nothing

in return from its parasitic inhabitants. The symbionts, however, repay their host by breaking down cellulose and other cow-indigestible materials, from which they produce short-chain fatty acids which the cow can utilize. Similar arrangements are found in herbivores of many kinds, from cows to cockroaches. But the host member of the pair stands to gain yet further rewards for hospitality rendered. Some at least of the symbionts can synthesize from very simple materials all the amino-acids and vitamins that they themselves require. These compounds become incorporated in the first instance into the substance of the symbionts, but these organisms are not immortal. When they die and undergo eventual autolysis, their essential amino-acids and vitamins become available to the host, at any rate in part. There can be little doubt that some herbivores depend largely upon their intestinal flora and fauna for supplies of essential accessory food materials, though it may be doubted whether supplies from these sources are ever sufficient by themselves.

Provided that their somewhat exacting nutritional requirements are fulfilled and that sufficient energy-yielding substances are available, heterotrophic organisms such as the mammals are capable of dismantling their food materials and rearranging the component parts in a very versatile manner. From its food proteins, for instance, an animal can build up the species-specific proteins that are characteristic of its own tissues and secretions; carbohydrates and even fats can be produced from the deaminated residues of superfluous amino-acids, carbohydrates can be converted into fats and so on. The first steps in this direction consist in the digestion and absorption of the food, and these are followed by storage or metabolism of the ingested materials.

By *digestion* we mean the hydrolytic breakdown of food materials, which consist pre-eminently of large molecules, into simpler compounds from which a given organism can build up its own tissues and food reserves. This definition is one that can be widely interpreted, for the food may be the food eaten by an animal, on the one hand, or, on the other, it may comprise the materials provided in a seed or an egg for the embryonic development of a plant or an animal. A seedling plant is as

heterotrophic as any animal until it reaches the daylight and can begin photosynthetic activities on its own account.

The seeds of plants contain considerable reserves of organic foodstuffs from which new plants can develop. At or before the time of germination, enzymes are present which may be said to have digestive functions, since they serve to dismantle the food materials into simpler components which the young plant then oxidizes or rearranges in its own characteristic manner. The seeds of the castor-oil plant, *Ricinus*, are rich in oils and contain a powerful lipase. Barley, which is rich in starch, contains powerful amylases and a maltase at germination. More is known, perhaps, about the enzymes of the jack-bean than of any other seed, if only because it has been so much exploited as a source of urease. A veritable army of enzymes has been described here, for apart from urease itself, this bean contains an amylase, a lipase and a pectinase, together with peroxidase, catalase and probably several others.

Among animals digestion may be accomplished in either of two main ways. The food may be phagocytically ingested and then intracellularly digested, or it may undergo extracellular digestion before being absorbed. Often both mechanisms are used side by side in one and the same animal. Intracellular digestion is probably more primitive than its extracellular counterpart, for phagocytosis is only possible for particles up to a certain order of size. That extracellular digestion arose as an adaptation to the necessity of breaking up relatively large food masses prior to absorption seems very likely, and it is probably significant in this respect that the peptidases involved in the extracellular digestion of proteins in the mammals are known to be qualitatively and quantitatively homospecific with the intracellular peptidases or kathepsins. In some animals it is possible to observe what seems to be a transitional process that is neither entirely intracellular nor wholly extracellular. In certain platyhelminth worms, for instance, the gut is lined with cells endowed with considerable amoeboid activity. When food is taken, these cells absorb water from it and swell up, sending out processes which form a syncytial network that fills the gut

cavity and enmeshes the food mass. Digestion takes place within the syncytium, which is later withdrawn. In some cases, however, a syncytium is formed but withdrawn before digestion has proceeded very far, and digestion continues even in its absence. Here it would appear that the function of the syncytium has been discharged once the enzymes it can provide have been liberated.

In many organisms, notably among the Protozoa and the sponges, phagocytosis followed by intracellular digestion is the only mechanism available for assimilation, but in other phyla it is not uncommon to find both the intracellular and extracellular modes of digestion used together. It usually appears in cases such as these that if the animal is carnivorous the extracellular enzymes are those which act upon proteins; if it is herbivorous the extracellular enzymes are those that act upon carbohydrates. Thus among the coelenterates, which are mainly carnivorous, proteinases are secreted by the walls of the coelenteron while non-protein materials are digested intracellularly. Similarly, the only extracellular enzymes found among lamellibranch molluscs, which are almost exclusively herbivorous, are amylases. The general disintegration of the food mass that results from the action of extracellular enzymes is usually facilitated by mechanical movements by the walls of the digestive cavity, so that the mass is eventually reduced to a particulate dispersion fine enough to allow of phagocytosis, and digestion is subsequently completed within the cells. But when we come to animals as complex and as highly specialized as the mammals we find that digestion is entirely extracellular. Indeed, the only remnant of the phagocytic systems which are so important among invertebrates is that found in the wandering scavenger cells of the reticulo-endothelial system.

Relatively little is known about the digestive processes of invertebrates, but the very large literature of the subject indicates, in a general manner, that animals as a whole are equipped with enzymes competent to break down fats, proteins and carbohydrates into their simple constituents. Proteinases, lipases and carbohydrases have been detected, either in the extracellular digestive juices or in the cells of the digestive glands themselves,

in a very large number of cases. It is not always possible to be sure that, because a protein-splitting enzyme is demonstrably present in the cells of a digestive gland, it necessarily has a digestive function. Intracellular proteinases have, nevertheless, been described having pH optima in rather strongly acid or weakly alkaline media, and thus resembling pepsin and trypsin. In such cases it is possible that the enzymes in question are concerned with digestion. However, other proteinases have been extracted which have optimal proteolytic activity in the region of neutrality, and, in cases like this, it is at least equally likely that their function is akin to that of katepsin.

The nature of the chemical operations involved in digestion appears to be substantially the same in all kinds of animals, whether digestion is intra- or extracellular. Most is known about these processes and the enzymes which catalyse them in the mammals, and the ensuing description of digestion will relate mainly to these animals.

It is not unusual to think of digestion as a process which is divisible into a series of nicely defined steps, each of which leads to equally nicely defined products. We find in the literature an abundance of statements to the effect that pepsin digests proteins thus far and no farther, that trypsin digests them farther to another definite point, and so on. While it is perfectly true that each enzyme, taken by itself, will carry out certain perfectly definite operations and cease acting when these have been accomplished, it must be remembered that digestion is not carried out in this manner. Food that has passed into the small intestine of a mammal, for example, is exposed to the simultaneous activity of all the pancreatic and intestinal enzymes, and its digestion is not separable into a series of discrete steps and stages but is, rather, a continuous process. The fact that the digestive secretions of animals have been resolved into a number of individual catalysts, each of which can be studied separately, has tended somewhat to encourage the step-by-step outlook on digestion as a whole. Perhaps the best way to check this tendency is to think of the enzymes involved in digestion, not as a mere collection of catalysts, but rather as an organized *system*

of catalysts, so ordered and regulated as to carry out a long and complex but nevertheless continuous process.

The products of digestion form the raw materials for the processes of *metabolism*, a general term used to cover all the chemical changes going on in the cells and tissues of living organisms. These changes may result in a chemical simplification of the starting material, in which case we speak of *katabolism*, or in an increase of chemical complexity, when we speak of *anabolism*. Katabolic changes are usually associated with the liberation of a larger or smaller part of the free energy of the starting material and are therefore said to be *exergonic*. A larger or smaller part of this energy may be harnessed by the organism and used for the performance of work of some kind, e.g. in locomotion or chemical synthesis. Anabolic processes, on the other hand, are usually *endergonic*, i.e. they are attended by an uptake of free energy. This energy may be drawn from concomitant katabolism or else from the external world.

Living organisms of every kind appear to be able to accomplish anabolism at the expense of katabolism, but the ability to carry out anabolic changes at the expense of external energy is the prerogative of autotrophic organisms. Thus, when we are studying processes of katabolism we should have constantly in mind the question, how much energy becomes available to the organism? We may also ask in what form it becomes available, and how it is converted into chemical, mechanical, electrical, thermal or osmotic work as the case may be. Similarly, when anabolic changes are being considered, we must inquire whence and in what form the necessary energy is forthcoming, and how it is transferred from its source to its intramolecular destination. These are important questions; questions which, moreover, have remained practically unanswered and unanswerable until very recent times, but at last we have a few clear indications on which we may hope to found a new knowledge of biological energetics.

Before going on to consider metabolism in detail it is desirable to examine the phenomena of digestion, taking our information mainly from the mammals. This task we shall attempt in the rest of this chapter.

DIGESTION AND ABSORPTION OF PROTEINS

Saliva contains no proteolytic enzyme, and the first phase of digestion takes place in the stomach under the influence of pepsin. Pepsin, it will be remembered, is secreted in the form of an enzymically inactive precursor, pepsinogen. This is activated by the hydrochloric acid of the gastric juice, which provides at the same time an acid medium of which the pH is about optimal for the action of pepsin. The latter, which acts more rapidly upon denatured than upon native proteins, opens up certain particular peptide links in its substrates, but whether or not it is able to complete its work depends a good deal on the consistency of the gastric contents. As soon as these have become liquid they are forced through the pyloric sphincter, whether peptic digestion has been completed or not.

After its passage through the pylorus the partially digested food mass, or chyme, is mixed with the pancreatic juice and the bile. Taken together, these secretions contain about enough free alkali to neutralize the acid that has come through from the stomach, and the pH of the intestinal contents is brought nearly to neutrality. It was formerly believed that the pH is about 8.5 at this stage, but more recent measurements show that it usually lies between pH 6.5 and 7.

Trypsinogen and chymotrypsinogen, activated by enterokinase and by trypsin respectively, yield trypsin and chymotrypsin. These enzymes continue the process of hydrolytic disintegration begun by pepsin and open up more peptide links, to produce peptide fragments much smaller than the original food proteins. Few free amino-acid molecules are produced at this stage. Carboxypeptidase, contributed by the pancreatic juice, and aminopeptidase, secreted by the intestine, take up the task of degrading the polypeptide fragments inwards from the ends, liberating amino-acid molecules one at a time until, when the dipeptide stage is reached, the substrates pass out of their range of specificity and into that of the dipeptidases of the intestinal secretions, and these complete the digestion. Eventually, therefore, the amino-acids which enter into the

composition of the food proteins are set free, absorbed into the portal blood stream, and carried away into the general circulation by way of the liver.

In the past there has been considerable discussion as to whether protein foodstuffs are, in fact, completely broken down into their constituent amino-acids before being absorbed. Some favoured this view, while others believed that so long as the protein has been reduced to some soluble form such, for example, as a mixture of peptones, the function of the digestive enzymes has been satisfactorily discharged. There is now evidence in plenty to show that the latter view is erroneous. In the first place it is unlikely that peptones are absorbed as such because, if peptones are injected into the blood stream of mammals, a condition known as 'peptone shock' results, but nothing comparable follows the consumption of a protein meal. Abef, using an ingenious technique known as *vividiffusion*, showed that amino-acids, but no protein fragments of larger size, can be detected in the blood leaving those regions of the gut from which absorption takes place. This he did by leading off the emergent blood through a series of collodion tubes immersed in warm physiological saline, and returning it then to the circulation. After this performance he was able to isolate several amino-acids from the saline medium and to detect the presence of a number of others by chemical means, but no trace of products more complex than the amino-acids could be detected. There is, moreover, a large increase in the concentration of free amino-acid nitrogen in the blood while absorption is taking place in a normal animal. It may also be argued, though the argument savours a little of teleology, that animals do in fact possess a series of enzymes capable of carrying digestion right through to the free amino-acid stage, and that these enzymes would hardly have been perpetuated in the course of evolution unless they were of some use to the organism. Finally, there is the telling fact that some students have acquired considerable fame for themselves by consenting over considerable periods to the replacement of their dietary protein by mixtures of purified amino-acids without, however, appearing any the worse for the experience.

DIGESTION AND ABSORPTION OF CARBOHYDRATES

Few animals are equipped with enzymes capable of attacking cellulose, although this polysaccharide plays a very large part in the nutrition of herbivorous animals. In these creatures the task of digesting cellulose is usually delegated to vast hordes of symbiotic micro-organisms (p. 189), and the useful products of their activity consist in the main of short-chain fatty acids. The mechanisms of this degradation are complex, if only because many different kinds of micro-organisms are involved. At the present time not a great deal is known about the details of the process, but they are being actively investigated.

Like cellulose, the so-called hemicelluloses (xylans, arabans, mannans, galactans, etc.) and fructofuranosans (such as the levan of grasses and the inulin of the Jerusalem artichoke and other Compositae) are not digestible by the enzymes of most animals, although they can be handled by symbiotic micro-organisms and probably yield products similar to those formed from cellulose.

The digestion of starch and glycogen is initiated by salivary amylase but, unless the eater follows the precept of Mr Gladstone and chews each mouthful of food quite an unbelievable number of times, little digestion takes place in the mouth. The food, more or less intimately mixed with saliva, is swallowed and passes on into the stomach. Although the optimal pH for salivary digestion lies very near to neutrality, the secretion of the strongly acid gastric juice does not put a sudden end to salivary digestion, because it takes time for the acid to penetrate into the food bolus. The consistency of the food mass is therefore an important factor. Eventually, however, the free acid of the gastric contents reduces the pH to a value at which the salivary amylase is inactive and is actually destroyed, but in the meantime starch and glycogen alike have been at least partly broken down to yield maltose and, if digestion is not yet complete, some dextrins.

The gastric juice itself contains no carbohydrase, but a notable concentration of free hydrochloric acid is present and contributes something to the digestion of carbohydrates containing

fructofuranose units. Fructofuranosides such as sucrose and inulin are hydrolysed with great ease and rapidity by warm, dilute mineral acids, and it is believed, therefore, that substances such as these undergo at any rate a partial hydrolysis during their stay in the stomach. The hydrolytic activities of the hydrochloric acid are cut short when the chyme passes through the pyloric sphincter and into the duodenum, where it encounters the strongly alkaline pancreatic juice and bile. Here the pH rises nearly to neutrality, and under these conditions the amylase of the pancreatic juice has almost its optimal activity. This enzyme finishes the work begun by the salivary amylase, and the conversion of starch and glycogen into maltose is completed.

Maltose, however, is only a transitory product, for it is rapidly hydrolysed under the influence of an α -glucosidase, the so-called 'maltase' of the intestinal juice. This secretion also contains a powerful glucosaccharase which completes the hydrolysis of sucrose, and a β -galactosidase, 'lactase', that deals with lactose. Ultimately, therefore, the carbohydrates of the food are resolved into their constituent monosaccharides and in this form they are absorbed from the gut. It is improbable that appreciable quantities of di- or higher saccharides are absorbed because, as is known from injection experiments, disaccharides present in the blood stream are largely excreted unchanged, and it is only in exceptional and probably abnormal cases that disaccharides appear in the urine.

The rates of absorption of different monosaccharides vary much more widely than might have been expected in view of the fact that all hexoses have the same molecular weight, while that of the pentoses is not very different. It follows, therefore, that the absorption of sugars from the gut cannot be explained in terms simply of diffusion. The same conclusion follows from the fact that glucose, for example, can be absorbed from very strong solutions, and therefore against a large osmotic gradient. Many experiments have been made to discover what mechanisms are involved in the absorption.

The rate of absorption can be determined by opening up an experimental animal such as a rat, and introducing a known

amount of the sugar to be studied into a loop of intestine, previously tied off at both ends. The animal is kept for a known length of time, and the contents of the intestinal loop are removed and analysed. The amount of sugar absorbed is then found by difference. While experiments carried out on these lines show that galactose and glucose are absorbed much more rapidly than other sugars, they are open to serious criticism. There is, in the first place, a definite possibility that direct damage may be done to the gut, and it is also possible that the anaesthetic that must necessarily be used may interfere with the normal processes of absorption. But other methods of investigation are possible and yield substantially the same results. Cori, working on unanaesthetized rats which he fed by stomach-tube, obtained the results shown in Table 9. Other workers, including Verzár and his co-workers, obtained substantially the same results, though rather different ratios have been found in different animal species.

TABLE 9. ABSORPTION OF MONOSACCHARIDES FROM
THE SMALL INTESTINE OF RATS
(After Cori)

Sugar	Relative rate of absorption
D-Galactose	110
D-Glucose	100
D-Fructose	43
D-Mannose	19
L-Xylose	15
L-Arabinose	9

Pentoses are absorbed at the same rate as indifferent substances such as sodium sulphate. Galactose and glucose are absorbed so much more rapidly than the rest that a special mechanism of some kind must be deemed to be involved in their case. Verzár believed that this consists in the phosphorylation of the sugars in the gut mucosa under the influence of a phosphatase which is demonstrably present in the cells. He showed that the selective absorption of glucose and galactose can be abolished by adding iodoacetate or phlorrhizin to the contents of tied-off intestinal loops, and that absorption in the intact animal is much delayed by previous injection of these drugs. Both phlorrhizin

and iodoacetate are known to be powerful inhibitors of fermentation and glycolysis, and in both these processes phosphorylation is known to play a fundamental part. Verzář therefore regards his results as evidence that phosphorylation is involved in the absorption of sugars from the intestine.

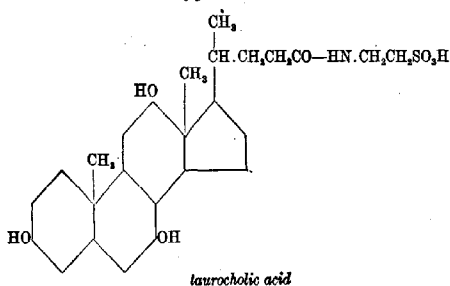
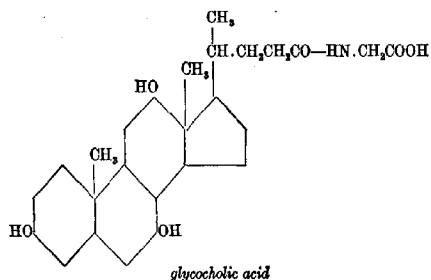
Verzář's observations might be regarded as strong evidence in favour of his contention if it were definitely known that iodoacetate inhibits phosphatases specifically, but there is no indication that this is the case. Iodoacetate is known to inhibit several of the enzymes concerned in glycolysis and in fermentation, but there is no reason at present to believe that it inhibits enzymes concerned with phosphorylation. It follows that the effect of iodoacetate upon the absorption of sugars such as glucose and galactose *may* be due to its action upon the gut phosphatase, but it does not necessarily follow that this is the case. It may equally well be true that iodoacetate acts upon other enzymic systems, upon which the maintenance of the normal physiological condition of the cells depends, and that its action upon the processes of absorption is only indirect. Verzář's phosphorylation hypothesis may possibly be right, but rigid proof of it is still lacking.

A point of interest may be added in passing. It is well known that phlorrhizin abolishes the reabsorption of glucose from the urine by the cells of the kidney tubule, as well as its specific absorption from the small intestine. The tubule cells, like those of the gut mucosa, contain a powerful phosphatase, and the facts suggest that both groups of cells carry out the work of absorption by essentially similar mechanisms.

DIGESTION AND ABSORPTION OF FATS

Saliva contains no lipase and, while the presence of a lipase in the gastric juice has been reported in a number of cases, the activity of the alleged gastric lipase at the pH of the gastric contents is such that it can be of little importance in digestion. Many authors believe that it must be regarded as pancreatic lipase that has regurgitated from the small intestine. But al-

though no appreciable digestion takes place in the stomach, the fats of the food are warmed and softened, if not actually liquefied. When presently the chyme is somewhat forcibly squirted into the duodenum there is a marked tendency for the fat to become emulsified, a tendency which is emphasized by the presence of the bile salts. The commonest representatives of this important group of substances are conjugated derivatives of cholic acid



with glycine and taurine. They are remarkable for their property of very greatly reducing the surface tension at fat/water interfaces, and for this reason not only facilitate emulsification but tend to stabilize an emulsion once it has been formed.

Until the beginning of the century it was generally believed that finely emulsified fat can be absorbed without previous digestion, but it appears that the bile salts alone cannot produce a sufficiently fine dispersion. Finer emulsions can be prepared

with the aid of sodium cetyl sulphate, a synthetic wetting agent, and if emulsions made in this way are introduced into the duodenum of rats, the dispersed fat particles are absorbed. Sodium cetyl sulphate inhibits the action of the digestive lipases, and we have therefore to conclude that direct absorption of unhydrolysed fats can, indeed, take place. Even paraffin can be similarly absorbed, provided only that it is sufficiently finely dispersed. The essential conditions for absorption are, according to Frazer, that the particles shall be less than 0.5μ in diameter and that they shall be negatively charged. Sodium cetyl sulphate gives emulsions in which these conditions are fulfilled, but does not occur naturally, while the bile salts do not yield particles less than about 2μ in diameter.

Frazer and his colleagues attempted to find natural emulsifying agents which could produce the degree of emulsification required for direct absorption under conditions similar to those which prevail in the small intestine. The substances studied were bile salts, cholesterol, a free fatty acid (oleic) and a monoglyceride (glyceryl monostearate), separately and in various combinations. Only with bile salts + fatty acid + monoglyceride was it found possible to obtain the necessary degree of dispersion.

But not all the food fat is absorbed in the emulsified form. Animals possess powerful lipolytic enzymes, which probably would not have survived unless they were useful to their possessors. The action of the pancreatic and intestinal lipases upon ordinary neutral fats disengages the fatty acids from their combination with glycerol one at a time, giving, at first, a mixture of free fatty acids with di- and monoglycerides—precisely the materials required for the emulsification of the remaining unhydrolysed fat. It may therefore be concluded that a part of the fat of the food undergoes digestion before being absorbed, and that the products of its digestion, together with the bile salts, facilitate the emulsification of the remainder, which is then absorbed without previous digestive hydrolysis.

Digestion is carried out in the small intestine under the influence of pancreatic and intestinal lipases. At the pH prevailing

in the small gut, the eventual products of hydrolysis are glycerol on the one hand and free fatty acids on the other. It was formerly supposed that the gut contents are alkaline (pH 8-9) in this region, and that the fatty acids are accordingly neutralized to form soaps. These substances are appreciably soluble in water, and their absorption seemed to offer no problems. More recent estimates put the pH at 6.5-7.5 however, and in this range soaps do not exist as such, but give rise to free fatty acids. Soaps, moreover, are powerful haemolytic agents, while ulceration of the colon following the use of soap enemata is not an unknown occurrence. Soaps, then, are probably not formed. Free fatty acids, by contrast with the soaps, are characteristically insoluble in water, and the manner of their absorption is therefore more problematical.

Bile salts probably aid the digestion of lipoid materials by facilitating their emulsification and so presenting the digestive lipases with a larger surface upon which to attack their substrates. But bile salts are not essential for digestion. In experimental animals in which the bile duct has been ligated, or in human subjects in whom the bile duct is occluded, e.g. by the presence of gall stones, fat is still digested, as is attested by the presence of free fatty acids in the faeces. Again, if a fat such as olive oil is introduced into a tied-off loop of intestine, with or without the addition of bile salts, no absorption takes place, but if a small amount of lipase is also added, the contents of the loop undergo digestion. But the oleic acid liberated is absorbed only if bile salts were introduced into the loop at the outset. Thus bile salts play some part in the absorption of free fatty acids, as well as in that of unhydrolysed fat.

This is attributed to the so-called hydrotropic action of the bile salts, i.e. their ability to form water-soluble complexes with fatty acids. This effect can be demonstrated readily enough by adding a solution of sodium glyco- or taurocholate to an aqueous emulsion of a fatty acid. If enough bile salt is added, the emulsion becomes water-clear. The simplest and smallest molecular complexes formed in this way are believed to contain one molecule of fatty acid and four of bile salt, but larger aggregates can be

formed with fatty acid : bile salt ratios of 2 : 7, 3 : 8, 4 : 9 and so on. The ability of these complexes to pass through a membrane will therefore be determined by the relative proportions of fatty acids and bile salts. If the fatty acid : bile salt ratio is low the particles will be small, and their ability to pass through the intestinal barrier will be proportionately greater. This hydro-tropic effect on the part of the bile salts is exerted upon other lipid materials such as cholesterol, but does not extend to unhydrolysed fats.

Many experiments were carried out by Verzár and his colleagues on the absorption of fatty acids, but in discussing them it is well to remember that Verzár himself was of the opinion that fats must be fully hydrolysed before they can be absorbed. In his experiments, which were carried out earlier than those of Frazer, use was made of tied-off intestinal loops. Oleic acid and

TABLE 10. ABSORPTION OF FATTY ACIDS
(After Verzár)

Contents of intestinal loop	Oleic acid absorbed in 6 hr.
Oleic acid + bile salt	29.3
Oleic acid + glycerol + bile salt	24.9
Oleic acid + phosphate + bile salt	10.3
Oleic acid + phosphate + glycerol + bile salt	48.9
Oleic acid + phosphoglycerol + bile salt	72.7
Oleic acid + phosphoglycerol + bile salt + iodoacetate	0

bile salts were introduced into the loops in all the experiments, together with the other substances indicated in Table 10, which is taken from his work. Neither glycerol nor phosphate alone leads to any acceleration of absorption, but if both are present together the rate increases two or three times. This suggests the possible formation of some compound of fatty acids with glycerol and phosphate, i.e. of a lecithin-like substance. Compounds of this type are soluble in water and might therefore be freely absorbed. In lecithin itself, it will be remembered, glycerol is present in combination with phosphoric acid, and the fact that fatty acids are absorbed nearly twice as fast in the presence of phosphoglycerol as when its components are present separately adds considerably to the probability that a phospholipoid of some kind is indeed formed.

Like the absorption of sugars, that of fatty acids is inhibited by phlorrhizin and by iodoacetate, but this does not prove that phosphorylation is involved (cf. p. 200). Nevertheless, the phospholipoid content of the blood is higher during the absorption of a fatty meal than it is at any other time, while the phospholipoid content of the intestinal lymph rises from a resting level of about 2.2 to about 7.5 mg. % while fat absorption is taking place. Furthermore, if an animal is fed with fat that has been 'labelled' with iodine or with heavy hydrogen, iodized or deuterated phospholipoids can be recovered from the gut mucosa while absorption is in progress. It seems likely, therefore, that fatty acids are transported into the cells of the mucosa, presumably in the form of water-soluble complexes with bile salts, and then condensed with phosphate and glycerol to form a substance resembling lecithin.

Phospholipoids of the lecithin group typically contain a nitrogenous base in addition to glycerol, phosphoric and fatty acids, but since some at least of the glycerol and phosphate required for the synthesis of the presumptive phospholipoid during absorption must probably be provided by the epithelial cells during normal absorption, it is not inconceivable that, if a nitrogenous base is required, it too can be furnished by the cells.

It appears, then, that a part of the food fat is digested by the pancreatic and intestinal lipases, and that the products of partial digestion, aided by bile salts, serve to emulsify the remainder so finely that it can pass through the intestinal wall without previous hydrolysis. How much of the total fat undergoes digestion and how much is absorbed directly is not certainly known. Probably it is reasonable to estimate that from one-quarter to one-third undergoes hydrolysis and that the rest, after emulsification, is directly absorbed.

Fat which is absorbed in the emulsified condition passes into the cells of the gut wall, in which it can be observed in the form of minute droplets which stain with dyes such as Sudan III and have in fact all the histological characteristics of neutral fat. From the cells these droplets, the chylomicrons, make their way into the lacteals and hence, through the lymphatic system and

the thoracic duct, into the blood stream, where they are responsible for the condition of post-absorptive lipaemia. That part of the fat which undergoes hydrolysis is ultimately resolved into glycerol and free fatty acids. The latter pass into the intestinal mucosa, apparently in the form of water-soluble complexes with bile salts, and here they appear to be built up into phospholipoids of some kind. These pass, not into the lacteals, but directly into the capillary blood vessels, hence into the portal blood stream and so to the liver.

CHAPTER VIII

GENERAL METABOLISM OF PROTEINS AND AMINO-ACIDS

FUNCTIONS AND FATE OF PROTEINS AND AMINO-ACIDS

PROTEINS constitute an indispensable article of food for all animals. We have abundant direct evidence from feeding experiments that the usual laboratory animals require supplies of certain amino-acids, notably tryptophan, lysine and histidine, not only for growth while the animal is young, but also for the maintenance of normal physiological condition during adult life. These *essential amino-acids* are only to be found in proteins, and protein foodstuffs are therefore indispensable. In some animals an indirect, secondary source of essential amino-acids is found in the tissue proteins of symbiotic micro-organisms inhabiting the gut but, although the value of this supplement may be considerable in some cases, we do not know if it can be sufficient alone. Evidence regarding the amino-acid requirements of invertebrate animals, unfortunately, is scanty. What information we have relates mostly to insects, but does not give grounds for supposing that their ingenuity in amino-acid synthesis is any greater than our own. Tryptophan, lysine and histidine seem to be essential for animals of every kind.

The naturally occurring amino-acids can be classified as in Table 11. Under the heading of essential amino-acids are some that can be replaced by other members of the essential group; thus tyrosine can be formed from phenylalanine, while cysteine can be produced if methionine is available. But the reverse is not possible. Provided enough phenylalanine is available to discharge the essential and characteristic functions of phenylalanine itself, *and* to provide at the same time enough tyrosine to

fulfil those of tyrosine, tyrosine itself need not be provided. Tyrosine, however, cannot discharge the functions of phenylalanine. Glycine, aspartic and glutamic acids, by contrast, need not be provided at all; these the organism can make for itself from non-protein materials, and of all the amino-acids that enter into the composition of the tissue and other proteins, there are not more than ten which the animal organism can produce by its own resources.

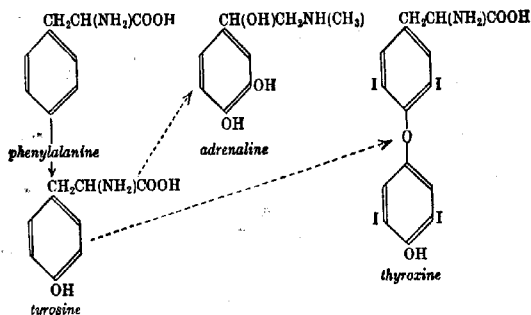
TABLE II. NUTRITIONAL STATUS OF AMINO-ACIDS

Non-essential	Essential	
	Irreplaceable	Replaceable
Glycine	Threonine	
Alanine	Valine	
Serine	Leucine	
<i>nor</i> Leucine	<i>iso</i> Leucine	
Aspartic acid	Methionine	Cysteine : cystine
Glutamic acid	Phenylalanine	Tyrosine
Proline	Histidine	
Hydroxyproline	Tryptophan	
Arginine	Lysine	
	Arginine	

The position of arginine, which figures as essential and as non-essential alike, calls for special comment. Adult rats remain alive and healthy, and young rats grow, on diets wholly devoid of arginine. But the growth of young rats on an arginine-deficient diet can be accelerated by the administration of arginine. A similar effect has been observed in chickens. It is therefore probable that, while the growing animal can evidently synthesize arginine to some extent, it cannot do so fast enough to keep pace with the requirements of optimal growth. It may be, indeed, that important substances other than arginine are synthesized so slowly as to limit the growth-rate of young organisms.

Amino-acids, essential and non-essential alike, are required for numerous processes. Quite apart from the special products to which particular individual amino-acids give rise, new tissue proteins must be synthesized, damaged or wasted tissues must be repaired or replaced, and normal supplies of enzymes and hormones must constantly be maintained. The formation of

adrenaline and thyroxine, for example, makes essential the provision of phenylalanine (or tyrosine), to which they are closely related and from which they are in all probability produced, as witness their respective formulae:



The production of a third hormone, insulin, makes heavy demands upon the supplies of essential amino-acids, for it is a polypeptide containing about 16 % leucines, 8 % phenylalanine, 12 % tyrosine, 4 % histidine, 3 % threonine, 2 % lysine and 12 % cystine.

Provided that the food proteins contain enough of all the amino-acids that are essential, the organism can probably make good any deficiency of the rest, although, in the ordinary way, it is not likely to be called upon to do so. The essential amino-acids are, on the whole, the least common, so that an adequate intake of these is necessarily attended by an adequate intake of the rest, so long as they are taken in the form of protein.

The elaboration of hormones, enzymes and other special products still goes on even during starvation, when it can only be done at the expense of the tissue proteins. Prolonged deprivation of protein therefore leads to emaciation and eventually to death. For some time before death ensues there is a small, fairly constant daily excretion of nitrogen, the magnitude of which may be taken as an index to the amount of protein being broken down

in the body. Death itself is heralded by a sudden extreme rise in the rate of nitrogenous excretion, known as the 'pre-mortal rise', and this begins when, the available carbohydrate and fat reserves of the tissues having been exhausted, the organism is left with only its tissue proteins as a source of energy production, so that a large-scale degradation of protein begins.

On a diet that contains very little protein it is possible for the daily intake of protein nitrogen to lie below the output. So long as output exceeds intake the organism, on balance, is the loser, and the deficit of protein nitrogen is withdrawn from the tissues. If, however, the protein allowance is gradually increased, a point is eventually reached at which intake just suffices to balance output. The organism is then said to be in a state of *nitrogenous equilibrium*. The amount of protein required just to attain this equilibrium condition in a given individual is therefore a measure of the *minimum protein requirement* of that individual, and since proteins are among the most expensive articles of food, this is a matter of economic as well as academic interest. Many workers have accordingly investigated the minimum protein requirements of the human organism, and the results obtained have been very variable indeed. Rubner and his colleagues put it at about 100-120 g. protein per diem for an average man, whereas Chittenden, using himself as the experimental animal, found that he could satisfy his personal requirements with only some 30-35 g. per diem, his health improving as a result of the experiment. These large differences do not, as might at first appear, merely reflect differences in individual requirements, but differences rather in the chemical nature of the food proteins chosen. These proteins must supply enough of the essential amino-acids, and no amount of protein, however great, that fails to accomplish this can suffice to establish nitrogenous equilibrium. Animal proteins are, on the whole, much richer than plant proteins in terms of their content of essential amino-acids, and it follows that smaller amounts of protein are required when meat, fish, eggs, cheese, milk and the like are chosen than when the food selected consists largely of cereals and pulses. The maize protein, zein, is notoriously deficient in tryptophan and in lysine, and

if zein is taken as the sole protein of a diet, nitrogenous equilibrium can never be established, no matter how much of it is consumed. Gelatin is similarly deficient in tryptophan and in phenylalanine, and, like zein, is a protein of 'poor biological value'.

The primary function of protein food is to supply the amino-acids needed for the growth, repair and general maintenance of the structural and catalytic machinery of living cells. If, as is commonly the case, the proteins of the food provide more amino-acid units than are required for the discharge of these primary and very specific functions, the excess can be degraded and made to subserve the secondary and far less specific function of providing fuel for the machine. If excess protein is taken the excess of nitrogen is eliminated, mostly in the form either of ammonia, urea or uric acid, within 24 hr. Proteins and amino-acids are not normally stored to any appreciable extent in the normal adult organism: nitrogen retention on a significant scale is only observed during periods of tissue growth, during childhood and pregnancy, for example, or during periods of protein replacement, as during convalescence after a wasting disease or after protein starvation. The non-nitrogenous residues are retained and serve to contribute to the stores of 'energy-producing' materials, i.e. carbohydrates and fats.

If a meal of protein is administered to a phlorrhizinized or diabetic animal an increased output of glucose and of acetone bodies is observed. Part of the protein must therefore be considered as convertible into carbohydrate derivatives and part into fatty metabolites. If the amino-acids are administered individually it is found that some are *glucogenic*, i.e. give rise to glucose, while others are *ketogenic*, giving rise to acetone or ketone bodies. The known fates of the amino-acids are summarized in Table 12. It will be noticed that certain amino-acids, including some of the essential group, give rise neither to glucose nor to ketone bodies. Their fate is unknown. It may be that they are incorporated into some sort of protein or peptide which serves as a temporary store, but we have little evidence for the existence of such a store.

TABLE 12. FATES OF AMINO-ACIDS ADMINISTERED TO A DIABETIC OR PHLORRHIZINIZED DOG

Glucogenic	Ketogenic	Fate unknown
Glycine (2)	Leucine (4)	<i>nor</i> Leucine
Alanine (3)	<i>iso</i> Leucine (4)	Lysine
Serine (3)	Phenylalanine (4)	Histidine
Threonine (3)	Tyrosine (4)	Tryptophan
Cysteine (3)		Methionine
Valine (3)		
Aspartic acid (3)		
Glutamic acid (3)		
Arginine (3)		
Ornithine (3)		
Proline (3)		
Hydroxyproline (3)		

Note. The numbers in brackets indicate the number of carbon atoms undergoing conversion in each case.

FATE OF α -AMINO-NITROGEN

Neither glucose nor the acetone bodies contain nitrogen. It follows, therefore, that, at an early stage in their metabolism, the amino-acids suffer the removal of their characteristic α -amino-group. In a typical mammal such as a dog, this α -amino-nitrogen ultimately appears in the urine in the form of urea. In birds, snakes and lizards, by contrast, the final end-product is uric acid, while in most aquatic animals ammonia is excreted instead. The urine of a dog starved of protein contains very little urea, but if a protein meal is taken, urea production soon begins and the protein-nitrogen of the food is almost quantitatively eliminated in the form of urea within 24 hr. or thereabouts. Now we are already aware that the food proteins are broken down by the digestive peptidases to yield the component amino-acids, and that it is in this form that the food proteins are actually absorbed into the blood stream. We have therefore to discover how, where, and in what form the α -amino-nitrogen is detached from the amino-acid molecules, and how urea is elaborated from the primary nitrogenous product.

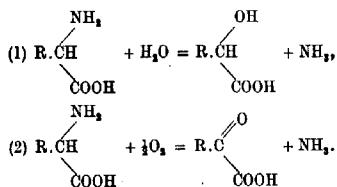
A partial answer to these questions is obtained by studying a hepatectomized animal or, alternatively, an animal with an Eck's fistula. An animal of this kind will survive for some days, but dies quickly if it is allowed to eat protein. At death, unusually large amounts of amino-acids are found in the blood,

but neither the blood nor the urine contains any urea in the case of a dog, or uric acid in that of a bird. Instead, ammonia is present, and it is from ammonia poisoning that the animal actually dies. These observations show (a) that the amino-groups of the amino-acids are split off in the form of ammonia, and (b) that the conversion of this ammonia into urea or uric acid, as the case may be, normally takes place only in the liver. The latter conclusion is confirmed by liver-perfusion experiments and by experiments on liver slices. If small concentrations of ammonia are perfused through a surviving liver or shaken with liver slices, urea is formed in the case of dog liver, while if a bird's liver is used, the addition of ammonia leads to the production of uric acid.

We shall discuss these processes separately, dealing first with the removal of the amino-groups, a process which is known as deamination.

DEAMINATION

The deamination of amino-acids with production of ammonia might be accomplished in either of two ways, both of which have been considered. It might be a hydrolytic (equation (1)) or an oxidative process (equation (2)):



Very little experimental evidence has ever been adduced in favour of hydrolysis as the mode of deamination in animal tissues, though it is known that hydrolytic deamination takes place in some bacteria. The vast bulk of evidence relating to animal metabolism is in favour of oxidative deamination. Early work involving liver perfusion supported this view, for if amino-acids were added to the perfusion medium, traces of the corresponding α -keto-acids could be detected later. Hydrolytic

deamination, on the other hand, would require the production not of the keto- but of the corresponding hydroxy-acids. Further evidence pointing to oxidative deamination was obtained in studies on alcaptonurics, but this is open to serious criticism (p. 176).

The most convincing work on this problem was that carried out by Krebs, who made use of the tissue-slice technique. Slices of various rat tissues were shaken under physiological conditions of temperature, pH, etc., in the presence of various amino-acids. After an hour or two the reaction mixture was deproteinized and the corresponding α -keto-acids were sought and found by taking advantage of the fact that they form very insoluble, characteristic 2:4-dinitrophenylhydrazones. Among mammalian tissues only liver and kidney deaminate amino-acids at all rapidly, and these tissues use more oxygen when they are deaminating than when they are not. These observations give general support to the view that the deamination of amino-acids is essentially an oxidative process.

Surviving liver and kidney deaminate both the naturally occurring L-series and the non-natural D-series of amino-acids, but not all amino-acids are attacked at the same speed. Table 13,

TABLE 13. RATES OF DEAMINATION OF AMINO-ACIDS
BY SLICED RAT KIDNEY TISSUE
(After Krebs)

Amino acid added	QO ₂	
	Without amino-acid	With m/100 amino-acid
Glycine	- 21.1	- 21.4
DL-Alanine	- 23.5	- 41.0
DL-norLeucine	- 17.4	- 23.2
DL-Aspartic acid	- 23.5	- 37.0
L-Glutamic acid	- 23.5	- 43.2
DL-Proline	- 18.5	- 37.8
L-Hydroxyproline	- 18.5	- 19.8
L-Lysine	- 21.1	- 25.4
DL-Valine	- 21.1	- 25.0
L-Tryptophan	- 18.9	- 13.5
L-Histidine	- 18.9	- 21.0
DL-Phenylalanine	- 21.1	- 23.3
L-Tyrosine	- 18.7	- 24.4
DL-Leucine	- 23.5	- 29.1
L-isoLeucine	- 23.5	- 21.7

which is taken from Krebs's original paper, illustrates the relative differences in the rates of deamination of a number of amino-acids, and it will be noticed that the non-essential amino-acids are deaminated most rapidly, the rarer, essential acids being attacked more slowly on the whole. The data of Table 13 show the rate of deamination in terms of the extra oxygen uptake resulting from the addition of the amino-acids to the kidney tissue. Simultaneous determinations of the decrease of α -amino-nitrogen and of ammonia production gave results which, on the whole, ran parallel to those obtained by measurements of the oxygen uptake.

To obtain strictly quantitative evidence in favour of equation (2) is more difficult. If liver tissue is used, the ammonia set free by deamination is converted more or less completely into urea, but this difficulty can be obviated by the use of kidney slices, which do not form urea. But in liver and kidney alike, the other product of deamination, the α -keto-acid, is liable to be further metabolized. This, Krebs found, could be prevented by the addition of arsenious oxide. Working therefore with kidney slices and in the presence of arsenite, he was able to demonstrate that, for every molecule of ammonia produced, an extra atom of oxygen was consumed and a molecule of the corresponding α -keto-acid formed. Essentially the same results were obtained with extracts prepared from an acetone powder of kidney tissue (Table 14).

TABLE 14. OXIDATIVE DEAMINATION OF AMINO-ACIDS
BY KIDNEY EXTRACT
(After Krebs)

Amino-acid added	Mol. $O_2:NH_3$: keto-acid
DL-Alanine	1:1.94:1.83
DL-Valine	1:2.08:2.20
DL- nor Leucine	1:1.85:1.85
DL-Leucine	1:2.42:2.28
DL-Phenylalanine	1:2.17:1.85

Krebs went on to seek information regarding the mechanism of the process by studying tissue extracts. Pulp preparations of liver and kidney alike act upon both the L- and the D-series of amino-acids, again in an oxidative manner, but as soon as the

pulp is appreciably diluted its ability to attack the naturally occurring L-acids disappears. The enzyme responsible for the deamination of the D-series, however, is resistant to dilution, and powerful preparations of the D-amino-acid oxidase can be made by extracting fresh, finely divided kidney tissue with water or buffer, centrifuging to remove the tissue debris, and treating the clear extract with 10 vol. of ice-cold acetone under ice-cold conditions. By filtering off the resulting acetone powder and drying it carefully, a stable preparation can be had which retains its activity for some weeks, and from which active enzyme solutions can be made by extraction with water or with phosphate buffer. Preparations made in this way deaminate all the amino-acids of the D-series with three exceptions: glycine, D-glutamic acid and D-lysine. Specific oxidases were later discovered which deal with glycine and D-glutamic acid respectively, but there is reason to think that lysine is never deaminated at all. The D-amino-acid oxidase has been extensively concentrated and finally isolated, and a more detailed description of its nature and properties will be found on p. 101.

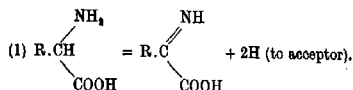
So far, however, no evidence had been obtained about the enzyme or enzyme systems involved in the deamination of the L-series of amino-acids. Numerous attempts have been made to obtain enzyme preparations which should act upon the naturally occurring amino-acids, but until quite recently only one such enzyme had been discovered.

This enzyme, which is present in the liver and kidney of mammals, is completely specific with respect to L-glutamic acid. Whereas D-amino-acid oxidase and the specific D-glutamic and glycine oxidases are capable of using molecular oxygen as their hydrogen acceptors, the L-glutamic enzyme requires either Co I or Co II, and is, in fact, a typical, coenzyme-specific dehydrogenase, now known as L-glutamic dehydrogenase. A further difference between the two cases lies in the fact that whereas the action of the oxidases seems to be irreversible, that of L-glutamic dehydrogenase is freely reversible.

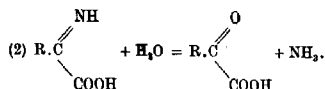
With the more recent discovery of an L-amino-acid oxidase in the liver and kidney of the rat, we are able to account for the

deamination of the majority of L-amino-acids, for the L-oxidase resembles the D-enzyme in being a true oxidase and relatively unspecific. It attacks all the mono-amino-mono-carboxylic amino-acids except glycine and those that contain a hydroxyl radical, but has no action upon the diamino- or dicarboxylic acids. How important this enzyme may be seems uncertain, for even in the rat it acts relatively feebly. It has been found in other animals in which, however, it is even weaker than in the rat.

Whether it is catalysed by the D- or by the L-amino-acid oxidase, or by L-glutamic dehydrogenase, *deamination is always oxidative and takes place in two stages*. In the first a pair of hydrogen atoms is transferred to the appropriate hydrogen acceptor and the corresponding α -imino-acid is formed:



The imino-acid then reacts, apparently spontaneously, with water to yield the α -keto-acid, together with ammonia:

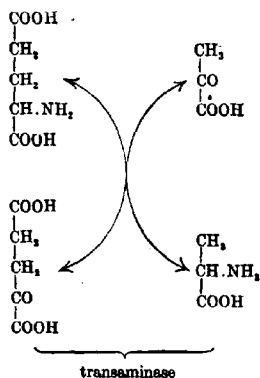


Special non-oxidative mechanisms are probably involved in the deamination of the hydroxy-acids, serine and threonine (pp. 231, 232), but the imino-acid is again formed as an intermediate.

TRANSDEAMINATION

In addition to deaminating enzymes, animal and plant tissues contain catalytic mechanisms which lead to the transference of amino-groups from amino-acids to other molecules. Braunstein and Kritzman found that if L-glutamic and pyruvic acids are added together to chopped liver or muscle tissue, the α -amino-radical of the glutamic acid is in part transferred to the pyruvic acid, so that α -ketoglutaric acid and alanine are formed. The system tends towards an equilibrium which can be approached

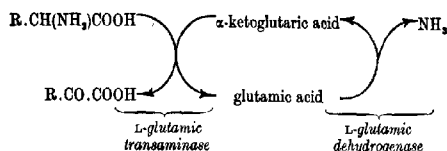
equally from either side, and the process, which for obvious reasons is referred to as 'transamination', was attributed by its discoverers to an enzyme which they named 'aminophorase'. This name has now been generally abandoned in favour of 'transaminase':



Enzymes of this kind seem to be very widely distributed in plant and animal tissues alike, and it is worth while to notice that, unlike the deaminating enzymes, they are not confined to liver and kidney among animal tissues, but are present also in brain, in muscle, and in heart, for example. They are specific, moreover, towards amino-acids of the natural L-series, and appear to be two in number. One of these transaminases is specific towards aspartic acid and the corresponding α -ketosuccinic (oxaloacetic) acid, and the other towards glutamic and the corresponding α -ketoglutaric acids. The glutamic enzyme seems to predominate in animal tissues and also in many plants.

Special emphasis must be placed upon the reversibility of transamination, for this process appears to be involved both in the breakdown of amino-acids and in their synthesis from non-protein sources. Before the discovery of L-amino-acid oxidase, Braunstein suggested that the deamination of L-amino-acids might involve transaminase. It was already known that α -keto-

glutamic acid is a common metabolite, arising as it does from carbohydrate as well as from protein sources. Under the influence of the glutamic transaminase the α -amino-groups of incoming amino-acids could be transferred to α -ketoglutaric acid to yield glutamic acid which then, under the influence of L-glutamic dehydrogenase in liver or kidney, would undergo deamination, α -ketoglutaric acid being regenerated and ammonia therefore set free:



Catalytic concentrations of α -ketoglutaric acid would be all that is necessary, if Braunstein's suggestion is correct, to catalyse the deamination of large amounts of L-amino-acids in tissues containing glutamic transaminase and the corresponding dehydrogenase. This same scheme could also explain why the dilution of a liver or kidney pulp leads to disappearance of its ability to deaminate the L-series of amino-acids: dilution would mean dilution of the α -ketoglutaric and glutamic acids which play the part of essential coenzymes in the Braunstein scheme.

Braunstein himself showed that L-alanine is oxidatively deaminated by cell-free liver extracts in the presence of catalytic amounts of glutamic acid. He was able also to build up a system capable of deaminating L-alanine by adding glutamic acid, Co I and purified L-glutamic dehydrogenase to partially purified samples of glutamic transaminase.

Opinion is still divided as to the extent to which this system can participate in the deamination of amino-acids in general. All authorities are agreed that the deamination of L-alanine, L-aspartic acid and L-glutamic acid can be accounted for by the action of glutamic transaminase plus L-glutamic dehydrogenase, but some have been unable to find evidence that other L-amino-acids can be similarly deaminated. Taken together with L-amino-acid oxidase, however, this system enables us to account for

the deamination of practically all the naturally occurring amino-acids, and it may yet prove that the transamination machinery is the more important of the two.

Since transamination is a reversible process, it follows that new amino-acid molecules can be synthesized at the expense of glutamic acid, provided that the appropriate α -keto-acids are available. At least three such α -keto-acids arise in the course of carbohydrate metabolism, viz. α -ketoglutaric, oxaloacetic and pyruvic acids. Glutamic acid can be synthesized from α -ketoglutaric through the reversed action of L-glutamic dehydrogenase, and can then act as amino-group donator for the synthesis by transamination of aspartic acid and alanine from oxaloacetic and pyruvic acids respectively. These mechanisms thus enable us to account for the production from non-protein sources of three of the few amino-acids that animal tissues are capable of synthesizing for themselves.

There can be little doubt that, on account of its reversibility, transamination plays a part of fundamental importance in the anabolism as well as in the katabolism of nitrogenous compounds. Foster and his co-workers kept a group of rats on a low protein diet and added heavy ammonium citrate to their food. The idea was to see whether the animals could utilize the heavy nitrogen, N^{15} , for the synthesis of nitrogenous compounds, and a low protein diet was used in order to increase the chances of its utilization. Later the animals were killed and creatine, glutamic acid, aspartic acid, histidine, arginine and lysine were isolated from the carcasses and analysed for heavy nitrogen. With a single exception in the case of lysine, all these compounds were found to contain significant quantities of N^{15} , showing that the heavy ammonia had, in fact, been used. The fact that N^{15} had found its way into all these substances serves to show (a) that ammonia can be used for the synthesis of amino-acids, and (b) that the α -amino-group of a given amino-acid is not simply a static feature of the amino-acid molecule, but that it must be undergoing a constant, dynamic interchange with other $-\text{NH}_2$ groups. That lysine fails to acquire N^{15} suggests that this particular amino-acid never undergoes biological deamination at all. The

N¹⁴ of arginine was found to be located entirely in the guanidine end of the molecule, suggesting that arginine, and probably ornithine too, therefore, do not undergo deamination. The close structural relationship between ornithine and lysine seems to be in accordance with this view. Foster's work provides important circumstantial evidence for the central importance of transamination in amino-group metabolism, and serves also to underline the importance of its reversibility. A reversible system in metabolism is not merely to be regarded as something that goes one way at one minute and the other way the next, but as a process that goes on simultaneously in both directions at one and the same time, so that the process as a whole is one that is essentially dynamic.

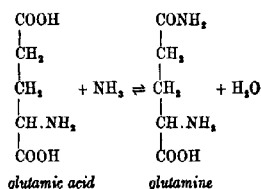
STORAGE OF AMINO-GROUPS

It will be realized that if amino-acid synthesis is to proceed in living tissues, amino-groups must be forthcoming. These might be taken from incoming amino-acids by transamination or, alternatively, ammonia itself might be utilized, if available. But ammonia is a very toxic metabolite, and concentrations of the order of 1:20,000 in the blood appear to be lethal to mammals (rabbits). Since non-essential amino-acids can be produced by animals, even under conditions of protein starvation, it follows that ammonia or amino-groups must in some way be stored in the tissues in an innocuous form. Further evidence that such a storage takes place is found in the fact that any tendency towards acidemia is counteracted in the mammal by the secretion of a more than usually acid urine, the excess acid present being neutralized more or less extensively by ammonia produced by the kidney. This process of ammonia production can still go on in animals temporarily deprived of protein foodstuffs.

No clue to the manner of this storage was found until, in the course of his experiments on deamination, Krebs observed that, of the ammonia produced by the deamination of added amino-acids, a part sometimes failed to put in an appearance. The disappearance of ammonia in liver tissue could be attributed to its conversion into urea, and it might have been thought that

some of this urea is later broken up by a tissue urease to furnish ammonia when the latter is required for amino-acid synthesis or for ammonia production by the kidney. In fact, however, there is not a shred of evidence for the occurrence of urease in mammalian tissues, although this enzyme is known to be present in the tissues of numerous invertebrates. If 'heavy' urea is administered to a mammal, preferably by injection so as to avoid possible bacterial intervention, the heavy nitrogen is quantitatively eliminated in the urine, still in the form of urea.

Even in the case of kidney tissue, which forms no urea, Krebs found that substantial amounts of ammonia could disappear on occasion, and he accordingly set himself the task of discovering its fate. He discovered that, in fact, ammonia can react with glutamic acid to form glutamine, the reaction being reversibly catalysed by an enzyme system that occurs in most animal tissues:



It is now known that many tissues, including even the blood, contain appreciable quantities of glutamine, and it would seem that glutamine can be synthesized by many tissues at times when ammonia is available, and broken down again to furnish ammonia when the latter is required.

Glutamine has long been known as an important constituent of plant materials, as also has the corresponding amide of aspartic acid, asparagine. The function of the latter as a storage depot for ammonia had been demonstrated many years earlier in studies of etiolated seedlings of the tree-lupin, *Lupinus luteus*. If the protein-rich seed of this plant is allowed to germinate in the dark, a seedling develops which is devoid of chlorophyll. Its development fairly soon comes to an end, and a study of the

nitrogen distribution in the plantlet shows that some 80% of the protein-nitrogen of the original seed has been converted into asparagine. The remaining 20% has been transferred to the tissue proteins of the seedling. If the seedling is now allowed access to light, asparagine begins to disappear and new protein is synthesized at the expense of the asparagine-nitrogen.

These changes can be accounted for if we suppose, as is undoubtedly true, that the processes of photosynthesis can give rise to α -keto-acids corresponding to all the amino-acids required for protein synthesis. It is necessary also to suppose, and this may not be so well justified, that transamination in the plant allows the transfer of amino-groups from aspartic acid to any α -keto-acid. In the etiolated seedling, oxaloacetic acid arises in the course of carbohydrate breakdown and can function as an amino-group receptor with respect to amino-acids formed by hydrolysis of the seed proteins. This allows the deaminated residues of the amino-acids to be utilized as fuel at a time when the organism is as yet unable to derive solar energy through chlorophyll and photosynthesis. The aspartic acid formed by transamination can react with more ammonia, produced by direct deamination of amino-acids, to yield asparagine, and the process can continue so long as the protein reserves hold out, subject only to the limitation imposed by the fact that the tissues, even of an etiolated seedling, contain some protein.

When presently the plant gains access to light, chlorophyll makes its appearance and photosynthesis begins. This leads to the production of α -keto-acids, among other substances and, as the concentration of these begins to increase, they begin to undergo transamination at the expense of aspartic acid so that new amino-acids are formed. The utilization of aspartic acid and the consequent fall in the concentration of the latter leads, in its turn, to the breakdown of more asparagine. This gives rise to more aspartic acid, which can be used for transamination, and to ammonia, which can be employed in direct amination of more of the α -keto-acids: alternatively, the free ammonia can be used to synthesize more aspartic acid from oxaloacetic, the latter arising as an intermediate in the oxidative breakdown of

carbohydrate. The whole cycle of events can be summarized as in Fig. 22.

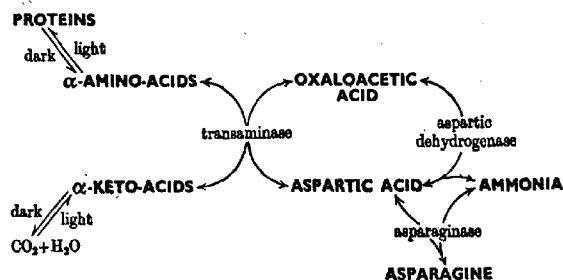


Fig. 22. Nitrogen metabolism in seedlings of *Lupinus luteus*.

Asparagine and aspartic acid seem, therefore, to fulfil in this plant a part parallel to that of glutamine and glutamic acid in many other plants and in animal tissues. Aspartic acid and its amide have long interested plant biochemists, and with good reason. In more recent times aspartic acid has been brought again into prominence by the claims of Virtanen, according to whom the primary amino-acid synthesized by the symbiotic root-nodule bacteria of leguminous plants is aspartic acid.

CHAPTER IX

SPECIAL METABOLISM OF THE AMINO-ACIDS

GENERAL

Glycogenesis and ketogenesis

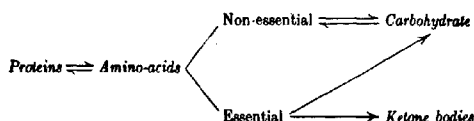
As has already been pointed out, certain amino-acids give rise to glucose if administered to diabetic or phlorrhizinized animals, or to glycogen in the livers of starving animals, and are accordingly said to be *glucogenic* or *glycogenic*. Others yield acetoacetic acid and the other ketone bodies and are said to be *ketogenic*, while a few give rise to neither.

If alanine is administered to a diabetic animal, all three of its carbon atoms are transformed into glucose. To state categorically that this glucose has been formed from the alanine is inaccurate, for it may only be that the deaminated residues of the amino-acid molecules were metabolized instead of glucose, and that an equivalent quantity of glucose or glycogen was therefore spared the fate of oxidation. Nevertheless, if the deaminated material replaces glucose or glycogen it presumably does so because it joins the metabolic pathway of glucose and glycogen, so that, for all practical purposes, it suffices to say that the amino-acid is convertible into glucose or glycogen.

Glucose and glycogen are formed from amino-acids by way of reaction chains which are often of considerable length. Many simple organic substances are known to be convertible into glucose or glycogen, and in some cases, e.g. lactic acid, pyruvic acid and glycerol, we know fairly precisely what the intermediate stages are. In other cases, e.g. the conversion of propionic acid into carbohydrate, we do not. In this chapter we shall content ourselves with tracing, where we can, the routes of conversion of amino-acids into substances which, like propionic, lactic and pyruvic acids, are known carbohydrate-formers. The rest of the stages are common to the metabolism of carbohydrates and

carbohydrate derivatives, and will be considered therefore in later chapters.

The amino-acids are classified as essential or non-essential according as they can or cannot be synthesized in the animal body. If the reader will refer to Tables 11 and 12 (pp. 208 and 212) several interesting points will be noticed. First, *all the non-essential amino-acids are glycogenic*, which probably indicates that their conversion into carbohydrate is a reversible operation. *Of the essential amino-acids only a few are glycogenic*, and the fact that they are essential probably indicates that the reaction chains through which they are transformed into carbohydrate include some step or steps that are irreversible. Thus carbohydrate can contribute to the synthesis of some amino-acids, but not of all. *All of the ketogenic amino-acids are essential*. They give rise to fatty metabolites, but the fact that they are essential, i.e. cannot be synthesized by the animal organism, shows that their conversion into ketone bodies is not a reversible performance. Thus fat, unlike carbohydrate, contributes little or nothing towards the synthesis of amino-acids or of protein. These general points may be summarized as follows:



The first step in the transformation of the amino-acids consists in the deamination or transdeamination of the amino-acids, the mechanisms of which have already been discussed. For convenience of reference the known modes of deamination of the naturally occurring L-acids are summarized in Table 15. It may be noticed that there is serious doubt whether lysine, ornithine and arginine undergo biological deamination (p. 220). It is known that serine, and perhaps threonine too, can be atypically deaminated by a non-oxidative enzyme present in rat liver (p. 231), which, in the absence of any other name, we may call 'serine deaminase'.

TABLE 15. DEAMINATION OF AMINO-ACIDS OF THE NATURALLY OCCURRING L-SERIES IN ANIMAL TISSUES

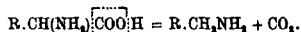
Amino-acid	L-Amino-acid oxidase	Transaminase system	Specific enzyme
Glycine	-	-	Glycine oxidase
Alanine	+	+	.
Serine	-	-	'Serine deaminase'
Threonine	-	-	† 'Serine deaminase'
Cysteine	+	-	.
Methionine	+	-	.
Valine	+	-	.
Leucine	+	-	.
isoLeucine	+	-	.
Aspartic acid	-	+	.
Glutamic acid	-	+	L-Glutamic dehydrogenase
Arginine	-	-	.
Ornithine	-	-	.
Lysine	-	-	.
Phenylalanine	+	-	.
Tyrosine	+	-	.
Tryptophan	+	-	.
Histidine	+	-	.
Proline	+	-	.
Hydroxyproline	? +	-	.

Note. The use of a negative sign indicates only that the amino-acid is not at present known to be deaminated by the system concerned.

Bacterial Attack and Detoxication

Bacteria of many kinds possess powerful and specific amino-acid decarboxylases, and although these do not fall within the scope of this book they must be mentioned here because they have considerable interest for animal metabolism. The alimentary canal of animals is always densely populated with bacteria at some region or other, and the activities of the members of this population lead to the production of materials which may be later absorbed by the animal itself. The extent of this absorption depends upon the region in which bacterial activity takes place. In herbivorous animals, of course, micro-organisms play a very large and important part in the digestion of the food, but even in animals that do not delegate their digestive operations to symbiotic bacteria, the metabolism of the micro-organisms has to be taken into account.

The bacterial inhabitants of the intestine have access to the food, or to the products of digestion of the food, among which amino-acids are included; and certain bacteria decarboxylate these to produce the corresponding amines:



Some of these products are intensely poisonous and, when they are absorbed into the animal's blood stream, undergo what is known as detoxication. This is accomplished by oxidation, reduction, acetylation, methylation, or in some other manner. Furthermore, by progressive bacterial degradation of the side-chains of acids containing aromatic rings, poisonous phenolic substances are formed, and these, like the amines, undergo detoxication in the animal body. We shall deal individually with individual cases, and we shall also comment upon the parts played by the amino-acids themselves, since several of them act as important detoxicating agents.

In addition to amino-acids, many other substances are employed in detoxication, and these may be briefly mentioned at this point. Acetylation is a common fate among aromatic substances containing $-NH_2$ groups, and the administration of aniline, substituted anilines, sulphonamide drugs and the like, is followed by their excretion, partly or wholly in the acetylated form. Amines are commonly oxidized, yielding harmless aldehydes or acids, by the amine and diamine oxidases of the tissues (p. 104). Phenolic substances are frequently excreted in conjugation with glucuronic acid or with sulphuric acid, the latter being probably derived from sulphur-containing amino-acids.

This book does not contain a special chapter on detoxication or, as it is often termed, protective synthesis; it has seemed more suitable to the author to dispense with such a chapter, since a large part of the subject can be dealt with under the special functions of individual amino-acids, while the foregoing remarks, together with a judicious use of the index, will fill in most of the gaps that will remain when the present chapter has been studied.

SPECIFIC METABOLISM

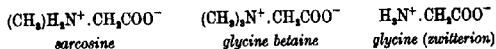
GLYCINE, $\text{H}_2\text{N} \cdot \text{CH}_2\text{COOH}$

(non-essential: glycogenic), is the simplest of the naturally occurring amino-acids and is the only member of the group that does not contain an asymmetric carbon atom. It gives rise on deamination by the specific *glycine oxidase* to glyoxylic acid,

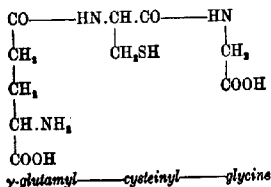


the metabolic fate of which is not fully known. We do not know how it undergoes conversion to carbohydrate in the animal body, but it is interesting to notice that there is a considerable delay between the administration of glycine to a diabetic or phlorrhizinized animal and the ensuing excretion of glucose in the urine.

Glycine gives rise to two methylated derivatives, *sarcosine*, a monomethyl glycine, and *glycine betaine*, in which three methyl radicals are present. The distribution and functions of these substances have been described elsewhere (p. 277). Glycine also

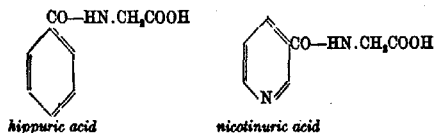


enters into the formation of *creatine* (p. 163). It occurs in combination in the peculiar tripeptide *glutathione*, which has the constitution of γ -glutamyl-cysteinyl-glycine: the peculiarity lies in the fact that it is the γ - and not the α -carboxyl radical of the glutamic acid unit that is engaged in forming the peptide linkage:



Glycine is also present in certain *bile salts* formed by the union of glycine with cholic and other bile acids (p. 201).

Finally, glycine plays an important part in the detoxication of aromatic acids such, for instance, as benzoic and phenylacetic acids, and also in that of nicotinic acid. These acids are excreted in the urine in the form of conjugates with glycine:



ALANINE, $\text{CH}_3\text{CH}(\text{NH}_2)\text{COOH}$

(non-essential: glycogenic), is the simplest of the optically active amino-acids. Two forms are known, one of which has the same spatial configuration as D-(-)-lactic acid and is now usually called D-alanine, while the other, with the spatial configuration of L-(+)-lactic acid, is known as L-alanine.

The prefixes D- and L- as nowadays applied to amino-acids give no indication of the effect of these substances upon polarized light: *they refer only to the configurational arrangement of the molecule.* It is possible, for example, to have a D-acid that is laevorotatory, and if in such a case it is necessary to indicate both the configurational nature *and* the optical properties of the compound, its name is prefixed by D-(-)-. This usage cuts sharply across the older terminology, in which *d*- and *l*- referred only to the sign of optical rotation. Consequently some amino-acids having the L-configuration, e.g. alanine, arginine and lysine, are described in the earlier literature by the prefix *d*-, and this custom is still used by some writers at the present time.

It is an interesting fact that, while all the naturally occurring amino-acids can exist in both the D- and the L-forms, the D-isomers occur only very rarely in nature; so rarely, in fact, that the prefix L-, which refers to the common forms, has been omitted throughout this book, except where it is necessary for the sake of clarity. Chemical synthesis of the amino-acids ordinarily yields racemic products, i.e. mixtures of the D- and L-forms. Biological synthesis, on the other hand, yields only the L-form

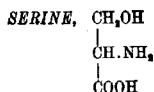
on account of the stereochemical specificity of the enzymes involved.

Another important general feature of the amino-acids found in proteins and simpler peptides is that they are members of the α -series, i.e. the amino- and carboxylic grouping involved in peptide formation are both attached to the α -carbon atom. To this rule there are but few exceptions. As we have just seen, there is an exception in the case of glutathione, a natural tripeptide in which a γ -carboxyl radical is involved in peptide formation. An exception of another kind is found in the occurrence of β -alanine,

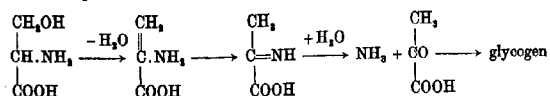


in the dipeptides *carnosine* and *anserine* (p. 288). β -Alanine also occurs in a member of the B_2 group of vitamins, viz. *pantothenic acid*.

Alanine yields pyruvic acid on deamination or transdeamination. The product is known to lie on the direct line of breakdown and synthesis of glucose and glycogen, and we shall deal with its intermediary metabolism in a later chapter. For our present purposes it suffices to point out that alanine, since it yields pyruvic acid on deamination, is convertible into glycogen or glucose.



(non-essential: glycogenic), is one of the less common amino-acids, and not much is known about its metabolism. It can be deaminated anaerobically by an enzyme ('serine deaminase') that is present in cell-free extracts of liver, to yield pyruvic acid, a known glucose-former:

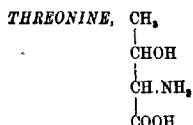


Probably the first of these reactions is catalysed by the enzyme, the remainder being perhaps spontaneous. It is not

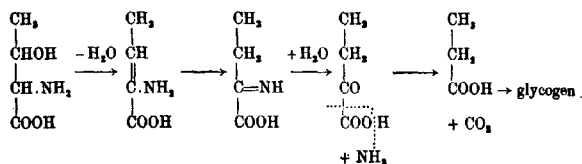
known, however, whether the conversion of serine into glycogen normally follows this or some other pathway.

Serine has been isolated from acid hydrolysates of casein in the form of the difficultly hydrolysable *phosphoserine*, suggesting that the organically bound phosphorus, which is a characteristic constituent of casein and other phosphoproteins, may find attachment to the protein molecule through the hydroxylic side-chains of serine residues.

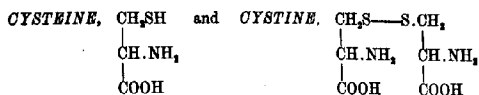
In addition, it has recently been shown that serine can exchange its hydroxyl radical for the sulphhydryl group of homocysteine to yield *cysteine* (p. 233).



(essential: glycogenic), like serine, is a hydroxylic amino-acid, but its functional importance and the mode of its conversion into carbohydrate are still unknown. It is possible that threonine is deaminated in the same manner as serine, giving rise to propionic acid and thence to glycogen:

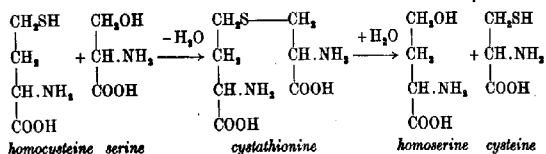


Threonine is one of the few essential amino-acids that are glycogenic, and it seems probable that, if the reactions suggested here correspond to reality, the reason that threonine cannot be synthesized in the body is that oxidative decarboxylation, a general reaction to which all α -keto-acids are liable, is irreversible.



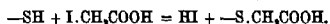
(essential but replaceable by methionine: glycogenic), are the commonest sulphur-containing amino-acids. How they are converted into carbohydrate is not known: conceivably they may yield up the characteristic —SH group to some other substance and give rise in this way to serine, which is also glycogenic.

That dietary cysteine and cystine can be replaced by *methionine* argues that they can be derived from it, and it has recently been shown that this is indeed the case. If methionine containing radioactive sulphur is administered to animals, radioactive sulphur can be recovered in cysteine and in cystine isolated from the tissues. The mechanism of the conversion has recently been discovered. Cysteine does not arise directly from methionine but from its demethylated product, *homocysteine*. If sliced rat liver is incubated with homocysteine in the presence of *serine*, cysteine is formed by a group-transfer reaction in which an —SH group changes place with an —OH. An intermediary reaction complex, *cystathionine*, is believed to be formed:

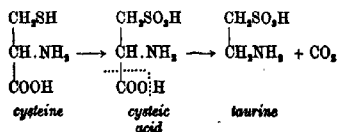


Cysteine and cystine are particularly important because of the ease with which a *pair of* —SH groups of cysteine can be oxidized to give the —S—S— bond of cystine, and vice versa. This property extends to many compounds into the composition of which cysteine enters. Thus it is present in *glutathione*, which we may represent as G.SH. This compound is very readily oxidized, e.g. by molecular oxygen in the presence of traces of heavy metals, to give the oxidized form, G.S—S.G, and it is upon this behaviour that the functional importance of glutathione appears to depend.

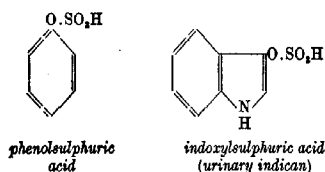
Linkages of the —S—S— type also play an important part in the intramolecular structure of *hair keratin* and other sclero-proteins. Hair contains about 7.3 % of cystine. It is believed that —S—S— bonds are formed between adjacent molecular fibres, and that the tensile strength and other mechanical properties of the hair fibre as a whole are largely due to these linkages. The —SH group is important also among *enzymes*, many of which are active only so long as their —SH groups are in the free state. If these are oxidized to give —S—S— bonds, catalytic activity disappears, but can be recovered by the addition of reduced glutathione. Although this is not by any means a universal property of enzymes, certain dehydrogenases in particular are reversibly inhibited by mild oxidation, and irreversibly by iodoacetate, which reacts with —SH groups in the following manner:



Cysteine is the mother substance of *taurine*, a compound which is very widely distributed, often in remarkably large amounts (p. 289). It is found in the bile of vertebrates as a conjugant in the *bile salts* (p. 201). Taurine is known to arise from *cysteic acid* through the agency of a specific cysteic acid decarboxylase, one of the few 'straight' decarboxylases known to occur in animal tissues (liver and kidney). Cysteic acid itself is believed to be formed from cysteine by oxidation, though the responsible enzyme has not so far been discovered. That the reaction takes place cannot be seriously doubted, however, for the administration of methionine containing radioactive sulphur can be followed by the isolation, not only of radioactive cysteine and cystine from the tissues, but by that of radioactive taurine also. The formation of taurine is therefore believed to take the following course:

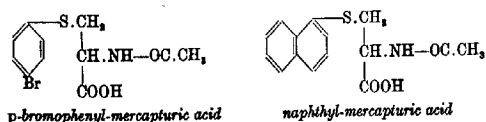


The oxidation of cysteine to cysteic acid may represent a step in the oxidative degradation of cysteine and cystine, the sulphur of which appears in the urine of mammals in the form of *inorganic sulphates*. Perhaps cysteine is also the source of the so-called *etheral sulphates* which appear in the urine following the absorption of phenolic substances into the body: their conjugation with sulphuric acid is one of several devices employed for the detoxication of phenols. Examples of etheral sulphates are the following:



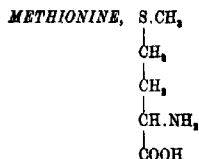
Unlike cysteic acid and taurine, these etheral sulphates are true sulphates and not sulphonic acids: the direct carbon-to-sulphur linkage characteristic of the sulphur-containing amino-acids is absent from the etheral sulphates.

Cysteine also contributes to the formation of *mercapturic acids*, which are the products of detoxication of certain aromatic substances. A classical example of this is seen in the fate of bromobenzene administered to dogs. The substance is eliminated in conjugation with *N*-acetylcysteine in the form of *p*-bromophenyl-mercapturic acid, a remarkable achievement on the part of an animal that is never likely to meet bromobenzene except through the medium of the laboratory. Naphthalene also is converted in part into a mercapturic acid:

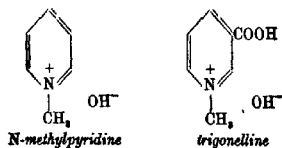


In addition to the inorganic and etheral sulphates, mammalian urine contains a third fraction, the so-called '*neutral sulphur*'. This comprises a mixed bag of sulphur compounds,

including traces of thio-alcohols (mercaptans) and mercapturic acids. Not much is known about this fraction, but there is no doubt that much of it arises from the sulphur-containing amino-acids. There exists, however, a rare condition known as *cystinuria*, in which the neutral sulphur fraction is very large and consists mainly of cystine itself. The administration of cystine to a cystinuric does not, however, increase the output of urinary cystine, showing that the cystine excreted is not directly derived from that of the food.

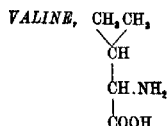


(essential), is not known to give rise either to glucose or to ketone bodies. Its main known function is that of a *biological methylating agent*. Thus it completes the biological synthesis of *creatine* by the transfer of a methyl group to glycocyamine (p. 163). Very possibly it is the methylating agent involved in the *detoxication* of pyridine and certain pyridine derivatives, including nicotinic acid, for pyridine itself is excreted in the form of *N*-methylpyridine, and nicotinic acid partly as trigonelline:

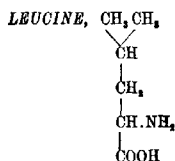
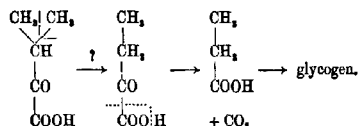


By undergoing demethylation, methionine is converted into *homocysteine*, the —SH group of which can be transferred to serine in the synthesis of *cysteine* (p. 233). On account of the ease with which homocysteine can be remethylated at the expense of the methyl radicals of choline, methionine plays a part of some importance in the *metabolism of phospholipoids* by con-

verting ethanolamine into choline and vice versa. Homocysteine can also be remethylated at the expense of glycine betaine and probably of sarcosine, but not at that of creatine.

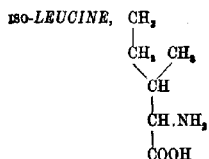
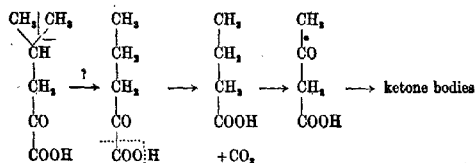


(essential: glycogenic), was formerly believed to be ketogenic, in common with the other branched-chain amino-acids. To explain its conversion into glycogen it is necessary to suppose that one of the methyl groups of the deaminated product can be removed, though whether this can be accomplished, and if so, how, we do not know. There is no reason to suppose that this group can be transferred to homocysteine to yield methionine, but the possibility seems not to have been investigated. Oxidative decarboxylation of the demethylated product would then give propionic acid, which is a known glucose-former:

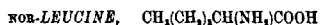
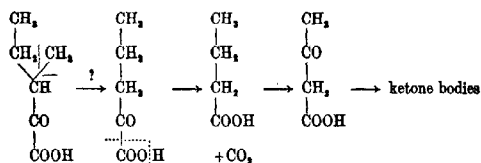


(essential: ketogenic). Its conversion into ketone bodies can be explained if we suppose, as in the case of valine, that one of the terminal methyl groups of the deaminated product can be removed in some way. If this were followed by the usual process of oxidative decarboxylation we should get butyric acid, which

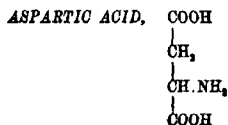
would be expected to undergo β -oxidation in the usual manner to give acetoacetic acid:



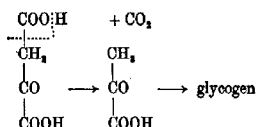
(essential: ketogenic), might be converted into acetoacetic acid by mechanisms similar to those postulated for valine and leucine, i.e. by the removal of a terminal methyl radical, followed by the usual further chemical changes:



(non-essential), is now known not to occur in proteins. It is not known to yield either glucose or ketone bodies: indeed, very little is known about the metabolism either of the leucines or of valine.

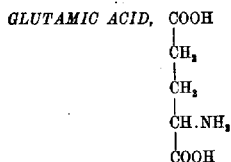


(non-essential: glycogenic), yields oxaloacetic acid on deamination or transdeamination. This product is somewhat unstable and undergoes slow, spontaneous β -decarboxylation under physiological conditions of temperature and pH. The liver contains an enzyme, β -carboxylase, which catalyses this reaction, of which the product, pyruvic acid, is known to give rise freely to glucose and glycogen:



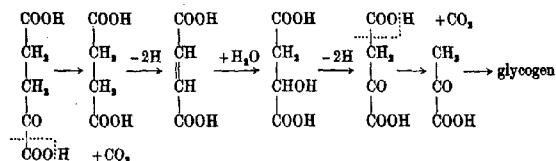
This rather rare process of β -decarboxylation contrasts sharply with the oxidative decarboxylation that is characteristic of α -keto-acids in general.

Aspartic acid can react with ammonia under the influence of the enzyme asparaginase to form *asparagine*, which is itself non-essential and glycogenic. This system plays an important part in the storage of amino-groups in the tissues of certain plants (p. 224). Finally, aspartic acid plays a central part in *transamination and transdeamination* in some animal and plant tissues.



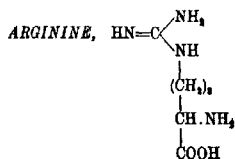
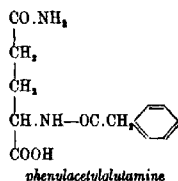
(non-essential: glycogenic), yields α -ketoglutaric acid on deamination. The product, an α -keto-acid, undergoes oxidative decarboxylation in the usual way, giving rise to succinic acid. Succinic is converted into fumaric acid by dehydrogenation, catalysed by succinic dehydrogenase, and fumaric acid, under the influence of fumarase, takes on water to give malic acid. Malic acid is dehydrogenated in its turn by the action of malic dehydrogenase

and Co I, to give oxaloacetic acid. The latter, on β -decarboxylation, yields pyruvic acid and hence gives rise to glycogen:



Glutamic acid itself plays a central part in *transamination* and *transdeamination*, and is a constituent of *glutathione*, while its amide, *glutamine*, occurs in considerable quantities in many plant and animal tissues, in which it represents a *store of amino-groups*. Glutamine, like glutamic acid, is non-essential and glycogenic, giving rise to glutamic acid and free ammonia under the influence of *glutaminase*.

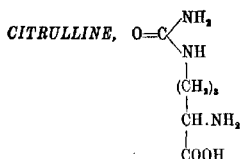
Glutamine plays a special part in the *detoxication* of aromatic acids, though only among Primates, according to present information. Phenylacetic acid is excreted in the form of a conjugate with glycine by most animals, but in man and the chimpanzee it gives rise to phenylacetylglutamine:



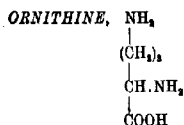
('half-essential', see p. 208: glycogenic), can lose its amidine group under the hydrolytic influence of *arginase* to yield *orni-*

thine, which is also glycogenic. How ornithine itself gives rise to glycogen we do not know: perhaps it is a long and somewhat tortuous process, for the rate at which arginine can be synthesized in the body appears to be slow enough to act as a limiting factor to the rate of growth of young animals kept on an arginine-deficient diet. Indeed, there are indications that arginine does not normally undergo deamination (p. 220).

Arginine can also part with its amidine radical by participating in group-transfer reactions, i.e. by *transamidination*. It is believed to play a central part in the synthesis of urea by the so-called *ornithine cycle* of Krebs. It occurs much more extensively among invertebrates than in vertebrates, for it forms the guanidine base of the *invertebrate phosphagen* (p. 283): in addition, arginine is probably the parent substance of some at least of the peculiar guanidine bases found in invertebrate tissues such, for example, as octopine and agmatine (Chap. XI).



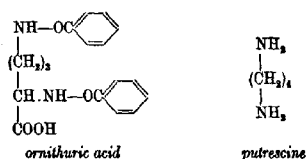
(non-essential: glycogenic), does not, so far as is known, enter into the composition of proteins, though it is thought to enter as an intermediary between ornithine and arginine into the '*ornithine cycle*' (p. 266).



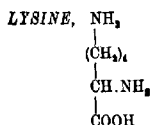
(non-essential: glycogenic), has not been isolated from protein hydrolysates, but arises from the action of arginase upon

arginine, and participates in the '*ornithine cycle*'. There is some doubt as to whether it undergoes biological deamination (cf. lysine).

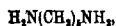
Among birds, ornithine discharges a special function in the *detoxication* of aromatic acids such as benzoic: the latter is excreted in the urine of birds in the form of dibenzoylornithine, or *ornithuric acid*:



By bacterial decarboxylation in the intestine and elsewhere, ornithine can give rise to the toxic diamine *putrescine*. Small amounts of this substance are absorbed by animals and are *detoxicated*, probably undergoing oxidation at the hands of diamine oxidase (p. 105).



(essential: not known to be glycogenic or ketogenic), is the next higher homologue of ornithine. It is said never to undergo deamination in the body (p. 220). Very little is known about the specific functions which, as an essential amino-acid, it must be presumed to discharge in the organism. Under the influence of bacterial enzymes it can yield *cadaverine*,



which, like putrescine, is highly toxic and can be oxidatively detoxicated by diamine oxidase.

PHENYLALANINE, $\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$



(essential: ketogenic), and

TYROSINE, $\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$

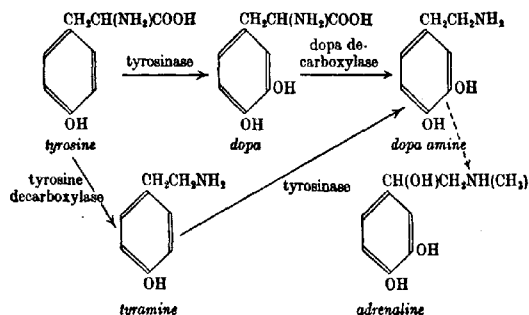


(replaceable by phenylalanine: ketogenic). Although these important amino-acids are known to be ketogenic, no satisfactory explanation of their conversion into acetoacetic acid is available at present. It is usually considered that the ring must be opened in the process: the side-chain contains only three carbon atoms, while four are required for the production of acetoacetic acid and the other ketone bodies. It may be presumed that phenylalanine is irreversibly convertible into tyrosine, but we have no certain knowledge of the mechanisms involved.

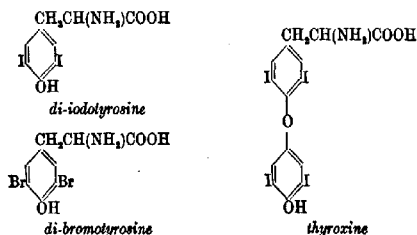
The metabolism of these aromatic acids goes astray in a group of interesting '*inborn errors of metabolism*'. A peculiar form of mental deficiency known as *imbecillitas phenylpyruvica* owes its name to the curious fact that the urine of the afflicted regularly contains small amounts of phenylpyruvic acid. In *albinism* the enzyme tyrosinase is completely lacking, and the dark-coloured *melanic pigments* (p. 98) are characteristically absent from the skin, hair, eyes and other usual situations. A single case of *tyrosinosis* has been reported, the urine in this disorder containing traces of tyrosine as a regular feature. *Alcaptonuria* is another disorder in which the metabolism of the aromatic amino-acids takes an abnormal course, and *homogentisic acid* is found in the urine (p. 177).

Phenylalanine and tyrosine are believed to be of particular importance in animal metabolism as the parent substances of two hormones, *adrenaline* and *thyroxine*. *Tyramine* can be formed from tyrosine by the action of a weak, specific tyrosine decarboxylase that occurs in the kidney and liver of mammals, and is thought to be an intermediate in the elaboration of adrenaline. If so, it must presumably be attacked by the monophenol oxidase component of tyrosinase to yield the amine corresponding to

dihydroxyphenylalanine ('dopa'). It is known, however, that dopa can be formed from tyrosine by the action of tyrosinase and, moreover, that there exists an apparently specific dopa decarboxylase which, acting upon dopa itself, could yield the same amine once more. Nothing is known about the remaining stages on the route to adrenaline, but the possible stages just described may be summarized in the following manner:

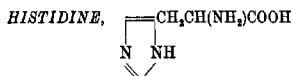


Thyroxine is a heavily iodinated derivative of tyrosine. It occurs, together with *di-iodotyrosine*, in the thyroid tissue of vertebrates. *Di-iodotyrosine*, together with the corresponding *di-bromotyrosine*, has also been described as a constituent amino-acid of the skeletal protein material of a coral *Gorgonia*, for which reason these halogenated tyrosines are sometimes entitled iodogorgoic and bromogorgoic acids respectively.



Phenylalanine and tyrosine give rise to a series of toxic products when submitted to bacterial attack. These include phenol,

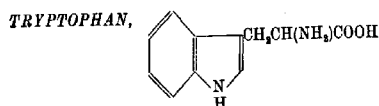
p-cresol, tyramine and phenylethylamine. The amines are probably *detoxicated* oxidatively by amine oxidase, and the phenols by conjugation with sulphuric acid.



(essential: neither glycogenic nor ketogenic), occurs in combination with β -alanine in the dipeptide *carnosine* (p. 288), which is present in the muscles of vertebrates of most kinds, though invariably absent from invertebrate tissues. The analogous compound *anserine* (p. 288) is similarly distributed and also contains β -alanine, but histidine is here replaced by its 1-methyl derivative.

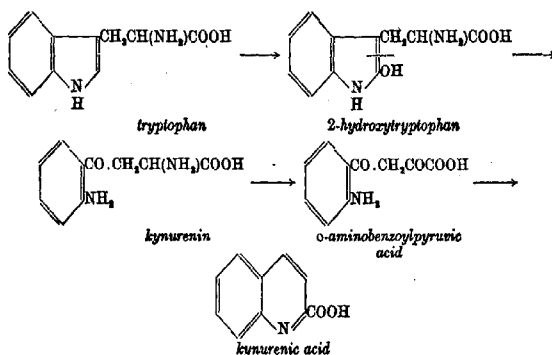
Comparatively little is known about the metabolism of histidine, though there are hints that it may be concerned in the *synthesis of purines* (p. 271). Animal tissues are known to contain a *histidase*, which is said to open the imidazole ring and yield, through a series of reactions, glutamic acid and succinic semialdehyde, both of which are strongly glucogenic, unlike histidine itself.

Histidine is the mother substance of *histamine*, being attacked by a specific *histidine decarboxylase*, traces of which are present in liver and kidney. Histamine can also be produced by bacterial activity in the intestine and elsewhere, and is *detoxicated* by a histamine oxidase, which may be identical with the diamine oxidase of animal tissues, though its distribution is a little peculiar.

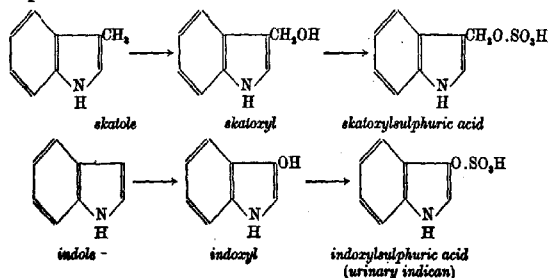


(essential: not known to be either glycogenic or ketogenic). This amino-acid gives rise, when administered in fairly large doses, to the excretion of *kynurenic acid*. This product is formed

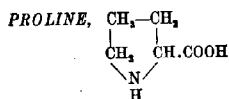
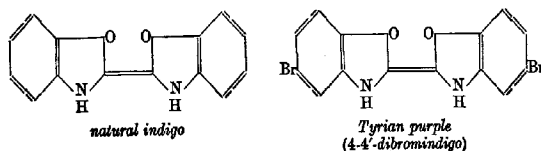
by way of *kynurenin*, apparently through the following reactions:



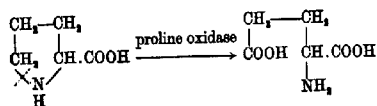
Bacterial decarboxylation of tryptophan leads to the formation of a poisonous amine, *tryptamine*, which is probably destroyed by amine oxidase, like other amines. Further degradation of the side-chain by bacteria is also possible and leads to the formation of a pair of foul-smelling compounds, *indole* and *skatole*; these are said to be largely responsible for the odour of faeces. Both undergo biological conversion into the corresponding alcohols, *indoxyl* and *skatoxyl*, which, if they are absorbed, are *detoxicated* by conjugation with sulphuric acid and excreted in the urine in the form of the corresponding ethereal sulphates:



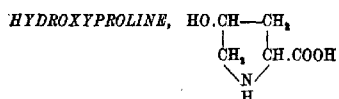
Finally, mention may be made of two natural pigments that are related to tryptophan, viz. *natural indigo*, from the woad and indigo plants, and the *Reyal* or *Tyrian purple* of the ancients, which may be prepared from a variety of marine gastropod molluscs, the classical source being *Murex* spp. Natural indigo arises from a glycoside of indoxyl that is present in the plant juices and undergoes decomposition when the tissues are bruised; free indoxyl is thus formed, and undergoes oxidative coupling in the presence of oxygen to yield indigo. Tyrian purple is similarly formed from a derivative, thought to be a mercaptan. of 4-brom-indoxyl:



(non-essential: glycogenic), is, strictly speaking, an imino- rather than an amino-acid. Little is known about its metabolism, apart from the fact that *proline oxidase*, which is present in kidney tissue, can open the ring and give rise to glutamic acid. The ring opening is not a hydrolytic but essentially an oxidative process. Since glutamic acid is glycogenic, the formation of glucose and glycogen from proline can be understood:



HYDROXYPROLINE



(non-essential: glycogenic), like proline, is an imino-acid. How it is converted into carbohydrate we do not know: conceivably it might first be reduced to proline and then attacked by proline oxidase. Alternatively, it might perhaps be attacked directly by proline oxidase to give γ -hydroxyglutamic acid, which is known to be glycogenic.

CHAPTER X

EXCRETORY METABOLISM OF PROTEINS AND AMINO-ACIDS

NATURE OF THE NITROGENOUS END-PRODUCTS

THE great bulk of all the nitrogen entering a typical animal arises from the α -amino-nitrogen of its food proteins. In mammals, at any rate, this is split off by deamination or transdeamination in the form of ammonia. Small quantities of ammonia arise also from other sources, such as the deamination of aminopurines and aminopyrimidines, but the great mass originates in the food proteins. The tissues have little capacity to store proteins or amino-acids as such, but appreciable amounts of ammonia can undoubtedly be stored in animal tissues in the form of the amide-N of glutamine. The storage capacity with respect to ammonia-N is, however, small compared with the average daily turnover of protein and amino-acid nitrogen. In plants, which have no excretory apparatus, larger quantities of nitrogen can be stored, either in the form of asparagine or glutamine or both, according to the species, but in animals, which possess efficient excretory machinery, the superfluous nitrogen is excreted. The excreta of animals contain a remarkable variety of nitrogenous substances of varying degrees of complexity.

Only among invertebrates do we find significant amounts of amino-acid nitrogen being excreted as such, and in certain invertebrate groups as much as 20-30% of the total ingested nitrogen may be excreted in the form of unchanged amino-acids (see Table 16). Whether this is due simply to leakage of amino-acids from the body fluids of these animals, or whether it indicates some sort of metabolic disability we do not know. In marine animals, at any rate, it is known that the surface membranes are permeable to water, to ions and to small molecules, and it is therefore possible that amino-acid molecules might be lost by diffusion to some extent.

EXCRETION OF NITROGEN

TABLE 16. NITROGEN PARTITION IN EXCRETA OF VARIOUS ANIMALS
(From data collected by J. Needham)

Phylum	Class and Order	Animal	Habitat	Ammonia	Urea	Uric acid	Amino-acids, creatine, etc.
Annelida	Chætopoda	Sea-mouse (<i>Aphrodite aculeata</i>)	SW	80.0	0.2	0.8	.
	Hirudinea	Leech (<i>Hirudo officinalis</i>)	FW	76.4	5.4	None	3.2
Gephyrea	Spunculoidea	Worm (<i>Sipunculus nudus</i>)	SW	50.0	9.7	None	16.6
Arthropoda	Crustacea	Crab (<i>Carcinus maenas</i>)	SW	67.8	2.9	0.7	8.7
		Spider-crab (<i>Maia squinado</i>)	SW	42.9	5.2	2.7	20.2
		Grayfish (<i>Astacus fluviatilis</i>)	FW	59.6	11.2	0.8	10.1
	Insecta	Silkworm (<i>Bombyx mori</i>)	T	.	None	85.8	.
		Clothes-moth (<i>Tinea pellionella</i>)	T	10.2	17.6	47.3	10.0
Mollusca	Gastropoda	Sea-hare (<i>Aplysia limacina</i>)	SW	33.5	8.7	4.6	13.0
	Lamellibranchiata	Land-snail (<i>Helix pomatia</i>)	T	13.7	20.0	10.7	6.0
		Clam (<i>Mya arenaria</i>)	SW	21.5	4.5	Trace	18.0
	Cephalopoda	Pond-mussel (<i>Anodonta cygnea</i>)	FW	63.0	.	.	.
		Octopus (<i>Octopus vulgaris</i>)	SW	41.7	15.0	.	20.7
		Cuttle-fish (<i>Sepia officinalis</i>)	SW	67.0	1.7	2.1	7.3
	Asteriidea	Star-fish (<i>Asterias rubens</i>)	SW	39.3	11.7	Trace	23.8
Echinodermata	Echinoidea	Sea-urchin (<i>Paracentrotus lividus</i>)	SW	28.1	7.5	1.0	28.0
	Holothuroidea	Sea-cucumber (<i>Holothuria tubulosa</i>)	SW	40.0	6.0	Trace	.

Vertebrate	Pisces: Teleostei		SW	36.8	16.2	0.0	15.8
		Goose-fish (<i>Lophius piscatorius</i>)	SW	36.8	16.2	0.0	15.8
		Sole (<i>Solea vulgaris</i>)	SW	53.0	16.6	.	13.8
		Sea-horse (<i>Hippocampus</i>)	SW	63.1	8.9	.	10.6
		Carp (<i>Cyprinus carpio</i>)	FW	56.0	5.7	0.2	7.0
	Dipnoi		FW	41.2	18.5	0.8	
	Elasmobranchii		FW	7.3	89.7	0.2	.
		Lung-fish (<i>Protopterus aethiopicus</i>)	SW		80.0	0.0	0.8
		Dog-fish (<i>Mustelus canis</i>)	SW		91.4		
		Dog-fish (<i>Scyllium canicula</i>)	SW	3.8			
		Electric ray (<i>Torpedo</i>)	FW/T	15.0	82.0	Trace	.
		Frog (<i>Rana temporaria</i>)	FW/T	3.2	84.0	0.4	.
		Frog (<i>Rana viridescens</i>)	FW/T	84.5		Trace	.
	Amphibia	Toad (<i>Bufo vulgaris</i>)	FW/T		84.5		.
		Turtle (<i>Chelonia mydas</i>)	SW	16.1	45.1	19.1	11.5
	Reptilia: Chelonia	Turtle (<i>Chrysemys picta</i>)	FW	39.0	18.8	18.8	
		Tortoise (<i>Testudo graeca</i>)	T	15.3	90.0	Trace	.
		Snake (<i>Boa constrictor</i>)	T	.		80.0	2.3
	Sauria	Snake (<i>Python</i>)	T	6.7		89.0	
		Grass-snake (<i>Tropidonotus natrix</i>)	T	Trace	Trace	80.0	.
		Lizard (<i>Lacerta viridis</i>)	T			91.0	.
		Horned lizard (<i>Phrynosoma cornutum</i>)	T	20.0	None	98.0	.
	Aves	Fowl (<i>Gallus domesticus</i>)	T	17.3	10.4	62.9	9.4
		Duck (<i>Anas</i>)	T	3.2	4.2	71.9	.
		Goose (<i>Anser</i>)	T	13.5		80.0	.
	Mammalia	Spiny ant-eater (<i>Echidna aculeata</i>)	T	6.9	81.2	None*	.
		Dog (<i>Canis vulgaris</i>)	T	3.0	88.0	1.0	.
		Cat (<i>Felis vulgaris</i>)	T	4.6	88.1	0.1	.
		Whale (<i>Balaena mysticetus</i>)	SW	1.5	99.0	3.0	.
		Bat (<i>Xantharpyia collaris</i>)	T	0.6	77.8	1.2	18.5
		Camel (<i>Camelus bactrianus</i>)	T	4.1	62.7	1.0	10.0
		Llama (<i>Lama guanaco</i>)	T	2.2	87.7	0.8	.
		Man (<i>Homo sapiens</i>)	T	4.3	87.5	0.8	.

All figures refer to nitrogen in terms of % of total nitrogen excreted.

SW = sea water; FW = fresh water; T = terrestrial.

* Uric acid is replaced by allantoin in almost all mammals (see pp. 100, 304).

Up to the present we have dealt mainly with the nitrogenous metabolism of mammals, chiefly because so much more is known about them than about any other group of animals. But there is reason to think that animals of every kind possess digestive enzymes capable of dismantling the food proteins completely to yield the component amino-acids. Although an excretion of unchanged amino-acids is observed among many invertebrates, the greater part of the ingested nitrogen is excreted in the form of ammonia, even among these animals. It is probable that all animals deaminate at any rate the greater part of their incoming amino-acids with production of ammonia. Whether the deaminating machinery is always the same, or even of the same general kind, we do not at present know, but the evidence points to a large-scale production of ammonia by deamination in all animals.

Now ammonia is a very toxic substance. Just how toxic it is can be appreciated if we consider some experiments carried out by Sumner, who injected urease into rabbits. The blood of the rabbit contains a small amount of urea, and from this urea ammonia was formed by the hydrolytic action of the enzyme. The animals died as soon as the concentration of ammonia in the blood rose to about 5 mg. per 100 ml., i.e. about 1 part in 20,000, a very high order of toxicity indeed. Death occurred before any change in the pH of the blood could be detected, and it is therefore probable that the toxicity of ammonia is due to some specific property of the ammonium ion rather than to the basicity of ammonium hydroxide. It is extremely improbable that death was due in these experiments to any toxic properties of the enzyme itself, for urease was also injected into birds, the blood of which does not contain urea, and in this case the animals were unharmed.

If we examine the excreta of many different kinds of animals, representing as many different phyla and classes as possible, we find that among the nitrogenous compounds present, some one compound always predominates. Over and above the traces of assorted odds and ends such as creatine, purine bases, betaines and the like, we find either ammonia, urea or uric acid accounting

as a rule for two-thirds or more of the total nitrogen excreted (see Table 16). In a few special cases, some compound other than these predominates, but such cases are rare and, in fact, animals as a whole may be divided rather sharply into three groups according as their main nitrogenous excretory product is ammonia, urea or uric acid. These three groups are respectively said to be *ammonotelic*, *ureotelic* and *uricotelic*. This discovery raises several important problems. First we must inquire why some animals are content to excrete their waste ammonia unchanged, and why others convert ammonia, the primary product of deamination, into secondary products in the form of urea and uric acid. Then we must ask why it is that, among animals that do elaborate these secondary products, some produce urea and others uric acid. Finally, we have to inquire into the mechanisms whereby these ultimate end-products are elaborated.

The nature of the predominant end-product in any particular case seems to be conditioned by the nature of the habitual environment of the particular organism, and the known facts are best explained on the supposition that *the conversion of ammonia to other products is an indispensable adaptation to limitation of the availability of water.*

If we consider the invertebrates first of all it may be said at once that they fall into a very large group of ammonoteles on the one hand, and a much smaller group of uricoteles on the other. Aquatic invertebrates, almost without exception, are ammonotelic. Ureotelism seems not to have been developed by members of the invertebrate phyla, while uricotelism is found only among terrestrial representatives of groups which, like the insects and the gastropod snails, have succeeded in colonizing the dry land. Animals living in water have at their disposal a relatively vast reservoir into which they can discharge waste ammonia, a relatively diffusible substance, without running any grave risk of being poisoned by their own excrement. Terrestrial invertebrates, on the other hand, are often hard put to it to find enough water for their essential needs, and the impossibility of disposing of ammonia fast enough to avoid toxæmia is overcome by the chemical conversion of ammonia, a very soluble and highly

poisonous material, into the insoluble and relatively innocuous uric acid.

The same general picture appears among the vertebrates, as the data of Table 16 make clear. Taking the fishes first it must be remembered that they fall into two main classes, the teleosts, or bony fishes, and the elasmobranchs, or cartilaginous fishes. Each of these classes is well represented in fresh and in salt water alike. To appreciate their position in the matter of water supply must involve a short digression.

The lives of aquatic animals are complicated by a factor which terrestrial creatures like ourselves have little reason to appreciate. The fact that an animal lives in water does not necessarily mean that it enjoys an unlimited supply of water. Among *marine invertebrates* the membranes bounding the body surface are, as a rule, more or less permeable to water and to small molecules. Ammonia formed in the body of such an organism can therefore escape comparatively readily by diffusion into the external environment. In *fresh-water invertebrates*, however, the boundary membranes are much less permeable: indeed, the main part of the body surface is impermeable to salts, and not very permeable even to water. This impermeability is necessary because the cells and tissues can only survive in the presence of considerably higher concentrations of salts than are present in the surrounding water, and surface impermeability is one of the devices employed to prevent leakage of salts out of the animal. But even so, the animal not only lives in water, it *breathes* in water, and this means that, in certain organs specialized for the purposes of respiration, the animal's blood comes into very close proximity to the surrounding water. In these respiratory organs, oxygen is taken into the blood and carbon dioxide eliminated, and the membranes of these organs have necessarily to be freely permeable to dissolved gases. It seems that the necessary degree of permeability to dissolved gases is inseparable from an appreciable degree of permeability to water. In the respiratory organs of a fresh-water animal, therefore, we find membranes that are permeable to water, though impermeable to salts; they are, in fact, approximately semi-permeable. On the outer side of these

membranes we have fresh water, which is virtually free of dissolved salts, and on the other lies the animal's blood, which contains on the average about 1 % of dissolved salts. For this reason there is a considerable osmotic force that tends to drive water into the animal from outside. The entry of this water leads to dilution of the salts of the blood, but aquatic animals possess elaborate salt-absorbing and excretory organs which enable them to turn out the unwanted water, while maintaining at the same time a constant internal salinity. A fresh-water invertebrate may therefore be pictured as having a constant, osmotically driven current of water passing through its body. Any ammonia formed in the cells and tissues will diffuse into the blood of such an animal and be carried away with the water in the form of a copious but very dilute urine.

The position is substantially the same for *fresh-water teleosts*. Ammonia formed in the tissues escapes rapidly and readily by way of the urine, and here, as in aquatic invertebrates, there is no danger of toxæmia due to the accumulation of ammonia. For *marine teleosts*, however, the position is considerably more difficult. The gill membranes and the mucous membranes of the mouth are appreciably permeable to water, as they are in the fresh-water forms. But sea water contains about 3 % of salts as against the 1 % or thereabouts present in the blood, and the osmotic flow of water in this case is therefore away from the fish, instead of towards it. Marine teleosts, therefore, although they inhabit a watery medium, are nevertheless poorly supplied with water. They lose water constantly to their environment and are liable to die of desiccation, unlike their fresh-water relatives, whose lives are constantly imperilled by the imminent threat of flooding.*

The nitrogenous excretion of marine teleosts has been investigated in a very ingenious experiment devised by Homer Smith. A wooden box is divided into two compartments by a watertight rubber dam pierced by a hole large enough to fit closely round the belly of a fish. The animal is placed in the apparatus, which

* For further information regarding the osmotic regulation of aquatic animals, see Baldwin, *An Introduction to Comparative Biochemistry*.

is filled with sea water, in such a way that the head and the gills are accommodated in one compartment and the tail and the excretory aperture in the other. After a suitable time, samples of water from either compartment are withdrawn and analysed for nitrogenous compounds, and it then appears that 80-90 % of the total nitrogen excreted is found in the forward compartment and must therefore have been excreted by way of the gills, only a small part of the whole being evacuated by way of the kidneys. About two-thirds of all the nitrogenous material excreted consists of ammonia, indicating that, although the fish has a comparatively poor water supply, it can, nevertheless, dispose of most of its ammonia by diffusion across the gill membranes, without previously converting it into any other nitrogenous compound. The remaining third is not present in the form of urea, nor yet as uric acid; indeed, it defied identification for some time, but turned out in the end to be trimethylamine oxide, $(\text{CH}_3)_3\text{N}\rightarrow\text{O}$. This, a practically neutral, soluble and innocuous material, is evidently formed from ammonia, which it partially replaces in the excreta of marine, but not of fresh-water, teleosts. It seems, then, that the marine teleosts eliminate ammonia as far as possible, but that the water supply is not sufficiently abundant to allow all the nitrogenous waste matter to be excreted in this form. Detoxication by the production of trimethylamine oxide is probably a trick which removes, or at least reduces, the danger of toxic ammonaemia in these fishes.

In support of the general validity of this argument it may be pointed out that, although not many fishes have been studied, there is no record of the occurrence of trimethylamine oxide in the tissues or in the excreta of fresh-water teleosts, though its presence in the tissues and excreta of marine forms has been abundantly confirmed.

The elasmobranch fishes present a slightly more difficult problem. *Marine elasmobranchs* produce and retain within their bodies large amounts of urea. Retention of urea in the blood and tissue fluids of these fishes is possible because the gills are impermeable to urea, while the kidney possesses a specialized mechanism that can control the loss of urea from the body.

Enough urea is always retained to keep up a concentration of 2-2.5 % of urea in the blood; over and above this concentration, urea is excreted, and the elasmobranch fishes are, in fact, ureotelic. The presence of so much urea in the blood raises the total osmotic pressure of the blood to a level slightly higher than that of the surrounding sea water, and these fishes therefore escape the constant loss of water which threatens the existence of the marine teleosts. Instead of losing water to their environment, they constantly receive an osmotically driven stream of water from the sea. By resorting to ureotelism, therefore, the marine elasmobranchs have not only protected themselves against toxæmia due to ammonia but, by retaining some of the urea they produce, have placed themselves in a very favourable position as regards water supply.

Like marine teleosts, the marine elasmobranchs convert a part of their waste ammonia into trimethylamine oxide. This suggests that they have at some time in the past experienced the same osmotic difficulties as confront the marine teleosts of the present day, and that they faced them in the same way, i.e. by converting a part of their waste ammonia into trimethylamine oxide. But subsequently, it appears, they evolved the still better trick of making urea, which is even less toxic than trimethylamine oxide, and, by retaining enough of it in their tissues, managed in the end to turn the osmotic gradient to their advantage instead of their detriment.

The *fresh-water elasmobranchs* are believed to be descended from their marine cousins, which they resemble in being ureotelic, although in their case the amount of urea retained is only of the order of 1 %. Thus, even among the fishes, an essentially aquatic group, we find ureotelism already well developed.

But no discussion of the fishes would be complete without some mention of the Dipnoi, or *lung-fishes*. These creatures inhabit swamps and rivers in tropical regions. During the hot season the water dries up, and the lung-fishes shut themselves up in cocoon-like structures in the mud to wait until the rains come. So long as water is available, these fishes behave like fresh-water teleosts and are ammonotelic. But during the period while they

lie dormant and cut off from the water, they switch over to ureotelism and, when the rains come and the rivers fill again, almost their first act on emerging is to excrete a mass of urea that has accumulated during their aestivation. This case is a particularly interesting one, since it constitutes a test case of the validity of our general hypothesis—that the detoxication of ammonia is essentially an adaptation to restriction of the water supply.

Going on now to the *Amphibia*, the frogs, toads, newts and the rest, we are in the company of animals which are able to spend longer or shorter periods away from the water. We should expect, in the terms of our hypothesis, that no animal could live long away from water without exposing itself to the hazards of ammonia-poisoning and, indeed, that the colonization of dry land could hardly have been begun until some mechanism had been evolved by means of which ammonia could be detoxicated. The *Amphibia* would be expected, then, to be either ureotelic or uricotelic. We get some very interesting evidence here by studying the humble tadpole. Tadpoles are aquatic and ammonotelic. Later, the tadpole undergoes a metamorphosis that changes it from a wholly aquatic animal into a true amphibian and, at the same time precisely, it also undergoes a chemical metamorphosis and abandons ammonotelism in favour of ureotelism. The adult frog, in common with the adult forms of other *Amphibia*, is ureotelic, and it seems as though, in the course of its development, it recapitulates some of the essential features of its evolutionary past. There is one special case that deserves mention; *Xenopus*, an animal that possesses the morphological features of a true amphibian, is exceptional in that although it undergoes metamorphosis, it remains throughout its life an aquatic, and an ammonotelic, organism.

The rest of the vertebrates are generally considered as having evolved from some primitive kind of amphibian stock which, we suppose, must have been ureotelic. Leading from the *Amphibia* we find two main, diverging lines of evolution, one leading to the mammals and the other to the reptiles and the birds. There exists among the reptiles one group, the *Chelonia* (tortoises and

turtles) some of which retain the ureotelic habit of their amphibian forebears, but the rest of the reptiles, the Sauria, together with the birds, have abandoned ureotelism in favour of uricotelism. The mammals, however, have continued in the amphibian manner and clung to the more primitive ureotelic habit.

Joseph Needham considers that the choice between ureo- and uricotelism is determined by the conditions under which embryonic development takes place. The case of the Chelonia has not been very thoroughly studied, but in certain species at least the eggs are laid in wet sand or in mud, and if urea were formed during development it could probably escape into the water of the immediate surroundings fast enough to avoid interference with the processes of ontogenesis. Ammonia, probably, could not escape fast enough under these conditions, and ureotelism presumably allows the embryo to get safely through its development. In one species the female lays her eggs in dry situations, but then goes to the trouble of wetting them very thoroughly with water from a supernumerary bladder, and this suggests that wetness of the environment must be an important factor in chelonian ontogenesis. There are indications, however, that in some species ureotelism is accompanied by uricotelism, and the whole problem calls for careful and thorough investigation.

The position is much clearer in the other ureotelic group, the mammals. There still remain a few egg-laying mammals, e.g. *Echidna*, and their eggs are incubated always in wet situations. Not much is known about the water relationships of these eggs, but it is, nevertheless, established that the adults are ureotelic. The rest of the mammals undergo embryonic development in intimate contact with the maternal circulation. Food materials diffuse from the maternal blood stream across the placenta to the embryo, and waste products can likewise diffuse across the placental barrier to be excreted by the maternal kidneys. The mammalian embryo, with the entire water resources and the excretory apparatus of the maternal organism at its disposal, has no need to do otherwise than remain ureotelic.

Conditions are very different in the eggs of the *snakes, lizards and the birds*. These eggs are laid with a supply of water just

sufficient to see them through development, and no more is to be had, for the eggs are surrounded by tough membranes or hard shells which are practically impermeable to water. In such a system the production of ammonia could be nothing short of disastrous. Urea would be a more suitable end-product if only because it is relatively harmless, but apart from the elasmobranch fishes, no organisms are known that can stand up to more than a very mild uraemia without more or less serious disturbances of normal physiological function. Needham has calculated that, if the waste nitrogen actually turned out during the embryonic development of the chick were converted into urea, the resulting uraemia, by human standards, would be sufficient to give the embryo a bad headache at the very least. 'In which case', as Needham says, 'natural selection would hardly have preserved it for our entertainment.' Embryos which develop in these closed-box, or 'cleidoic', eggs solved their problems by the conversion of waste ammonia, not into urea, but into uric acid, and the habit of uricotelism which they acquire during embryonic existence persists into, and throughout, their adult life. Whereas urea is a very soluble compound, the excretion of which requires a comparatively liberal supply of water, uric acid, which is almost equally innocuous, is exceedingly insoluble and can simply be dumped in the solid form. It is carried away from the embryo proper and deposited in a little membranous bag, the allantois, the contents of which include solid nodules of uric acid at the end of development.

Finally, mention may be made of a case of chemical recapitulation comparable to that already mentioned in the case of the frog. Needham has studied the nitrogenous excretory products of chick embryos at different stages throughout development. His results, which are shown graphically in Fig. 23, show that at the very beginning of development the chick produces ammonia, like an aquatic animal. This is quickly switched off in favour of urea, the embryo behaving for a time like an amphibious animal. Finally, the chick appears in its true colours, as a truly terrestrial, uricotelic organism, developing inside a cleidoic egg.

To summarize, we may make the following statements. Among invertebrates, the ammonotelic type of metabolism is found in aquatic animals; ureotelism appears not to be employed, while uricotelism is confined to organisms that have become adapted to life under terrestrial conditions. Among vertebrates, ammonotelism is confined to animals that are entirely aquatic and, even among these, ureotelism and the formation of trimethylamine

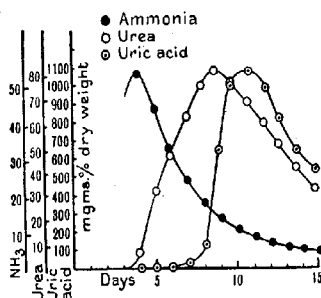


Fig. 23. Nitrogenous excretion of developing chick embryo (after Needham).

Maximum of	Days
Ammonia	4
Urea	9
Uric acid	11

Note differences in the scales.

oxide have been exploited by animals which, though aquatic, experience considerable shortage of water. With the conquest of the land, ureotelism appears to have been generally adopted and is still found to-day among the Amphibia. It has been retained by a group of reptiles, the eggs of which develop under conditions of comparative humidity, and in the mammals, whose embryos have the water of the maternal blood-stream at their disposal. The dry-living reptiles, together with the birds, have abandoned ureotelism in favour of uricotelism, a change which is associated with embryonic development under the conditions of acute water shortage implied by the cleidoicety of the egg. Thus the detoxication of ammonia by conversion to urea and uric acid appears in every case to be intimately associated with limitation of

water supply. A more pictorial form of summary is given in Table 17.

TABLE 17. NITROGEN EXCRETION OF VERTEBRATES
IN RELATION TO WATER SUPPLY

Group	Environ- ment	Water supply	$(\text{CH}_3)_2\text{N} \rightarrow \text{O}$	NH_3	Urea	Uric acid
Pisces:						
Teleostei	FW	Abundant	-	+	-	-
	SW	Poor	+	+	-	-
Elasmobranchii	FW	Abundant	-	-	+	-
	SW	Good	+	-	+	-
Dipnoi	FW	Abundant	-	+	-	-
	T*	None	-	-	+	-
Amphibia:						
Urodela: (<i>Xenopus</i>)	FW	Abundant	-	+	-	-
Anura: Tadpole	FW	Abundant	-	+	-	-
Frog	FW/T	Good/poor	-	-	+	-
Reptilia:						
Chelonia	FW/T	Poor	-	-	+	±
Sauria	T	Poor	-	-	-	+
Aves	T	Poor	-	-	-	+
Mammalia	T	Poor	-	-	+	-

Key. FW = fresh water; SW = sea water; T = terrestrial.

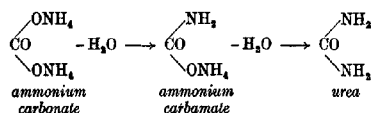
* During aestivation.

SYNTHESIS OF THE END-PRODUCTS: UREA

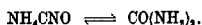
Most of the early work on the biological synthesis of urea was, not unnaturally, carried out on mammalian materials. It will be recalled that hepatectomy in the dog leads to cessation of urea production, an observation that points to the liver as the sole seat of urea synthesis in the mammalian organism. Work with perfused mammalian liver confirmed this conclusion, for a synthetic formation of urea from added ammonia was readily demonstrated. Little was known about the mechanisms of the synthesis for many years, but several theories were expounded, two of which have been widely held.

According to the classical view, ammonia reacts with carbon dioxide in the blood to form ammonium carbonate which, under the influence of the liver, was believed to undergo

two successive dehydrations to yield ammonium carbamate and finally urea:

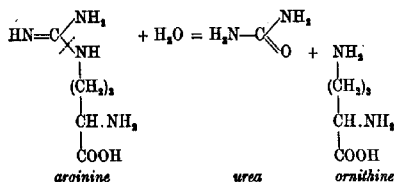


This view was eventually abandoned, numerous attempts to demonstrate urea production from ammonium carbamate having failed. A second theory was based upon the fact that the oxidation of nitrogenous organic substances under laboratory conditions often gives rise to traces of cyanic acid. If cyanic acid were similarly formed under biological conditions it could react with ammonia to form ammonium cyanate which, it was supposed, could subsequently undergo intramolecular rearrangement, after the manner of the classical preparation accomplished by Wöhler, to yield urea:



This scheme, too, was abandoned. Not only is cyanic acid toxic, but little evidence was forthcoming in favour of the supposed biological conversion of ammonium cyanate into urea.

The existence of a urea-producing enzyme in liver was suspected at the end of the last century, following the discovery that, if liver tissue is allowed to autolyse, urea is produced. Kossel and Dakin showed that this urea originates in arginine (set free by the autolysis of the tissue proteins) under the influence of a hydrolytic enzyme which they called arginase, and which catalyses the following reaction:



This enzyme can be extracted with water, saline, glycerol, etc., and has been studied extensively.

It was Clementi who first drew attention to the striking fact that, while *arginase* occurs in high concentrations in the liver of *ureotelic animals*, it is present in traces at most in the liver of those which are *uricotelic* (Table 18). It was already clear that *arginase* could be held responsible for the production of some of the urea excreted by mammals, inasmuch as *arginine* occurs in considerable amounts in most proteins. *Arginine* arising from the food proteins could therefore account for some, though not by any means for all, of the urea formed.

TABLE 18. DISTRIBUTION OF ARGINASE IN LIVER AND KIDNEY OF VERTEBRATES

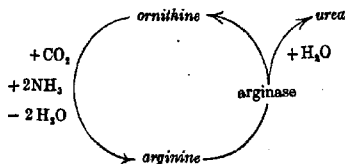
(Data from Clementi)

Class	Species	Liver	Kidney
Mammalia	Dog	+	-
	Ox	+	-
	Pig	+	-
	Guinea-pig	+	-
	Rat	+	-
	Monkey	+	-
	Man	+	-
Aves	<i>Gallus domesticus</i>	-	+
	<i>Columba livia</i>	-	+
	<i>Turtur turtur</i>	-	+
	<i>Fringilla cloris</i>	-	+
Reptilia:			
Chelonia	<i>Emys europae</i>	+	-
Sauria	<i>Lacerta agilis</i>	-	-
	<i>Anguis fragilis</i>	-	-
	<i>Coronella austriaca</i>	-	-
Amphibia	<i>Bana esculenta</i>	+	-
	<i>Bana temporaria</i>	+	-
Pisces:			
Elasmobranchii	<i>Torpedo ocellata</i>	+	.
	<i>Raja clavata</i>	+	.
Teleostei	<i>Perca fluviatilis</i>	+	-
	<i>Abramis brama</i>	+	-
	<i>Barbus fluviatilis</i>	+	-

Later, Krebs, using the tissue-slice technique, showed that surviving slices of rat liver can convert added ammonia into urea, thus confirming earlier observations made by the perfusion method. When amino-acids are added to the slices, the ammonia set free by deamination undergoes approximately quantitative

conversion into urea. Different amino-acids are deaminated at different rates, as we have already noted, and the rate of urea formation from amino-acids therefore varies from one to another. In one case, however, the rate of synthesis exceeded all expectation. It was to be anticipated that when arginine was added, not more than one and a half molecules of urea could be formed from each molecule of arginine used, one arising from the amidine group under the influence of the liver arginase, and a half from the α -amino-group, assuming that the latter is removed by deamination in the usual way. In fact, however, ten or more molecules of urea were found for each molecule of arginine. This suggested that arginine must act catalytically in the synthesis of urea.

Following up this clue, the effect of ornithine also was investigated, and again it was found that the rate, as well as the amount of urea synthesized from added ammonia, was greater than could be accounted for. Krebs therefore concluded that ornithine must react with ammonia and carbon dioxide to form arginine which, under the influence of the liver arginase, gave rise to urea, ornithine being regenerated and used over and over again as a carrier, thus:



The formation of arginine from ornithine must evidently be a complex process, and Krebs sought to discover possible intermediate compounds. It was known that there exists in the water-melon, *Citrullus vulgaris*, a substance which, chemically speaking, lies midway between ornithine and arginine. This substance, known as citrulline, has been tested, and evidence has been obtained that citrulline, like ornithine and arginine, can act catalytically in the synthesis of urea from added ammonia, though the results obtained with citrulline are less dramatic.

Krebs has therefore proposed the so-called 'ornithine cycle', the steps of which are illustrated in Fig. 24. This scheme explains the catalytic behaviour of ornithine, citrulline and arginine and, involving as it does the participation of arginase as an integral part of the system, provides a rational basis for the empirical rule enunciated by Clementi, i.e. that arginase is always present in the livers of ureotelic animals.

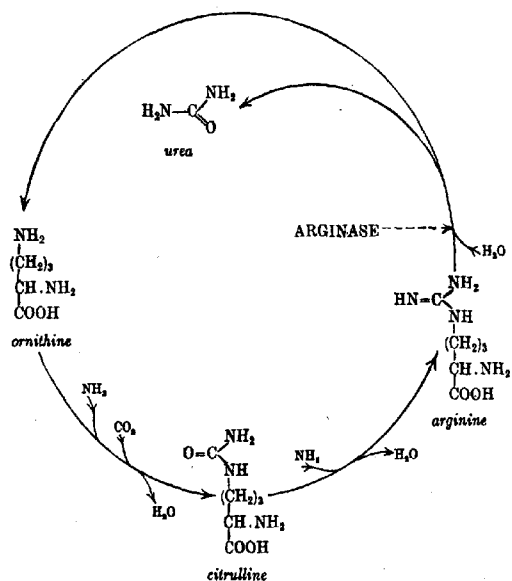


Fig. 24. The ornithine cycle.

The validity of Krebs's conclusions has been seriously questioned by a number of workers, to whose results we must refer shortly. In the meantime, certain points must be noticed in connexion with his original observations. The synthesis of urea from ammonia does not take place, even in the presence of ornithine, unless the liver cells are intact, and some oxidizable

material such as glucose or lactate is available. The dependence of the synthesis upon intact cellular architecture might be attributed to a breakdown of some one or other of the enzymes concerned, or to the loss of some essential coenzyme, when the cells are disrupted. The necessity for the provision of some kind of oxidizable material may be attributed to the fact that, being as it is an endergonic process, the synthesis of urea requires the provision of energy from some concomitant oxidative reaction.

Krebs's original work on rat liver has been extended to the livers of other ureotelic animals, and it has now been shown that ornithine acts catalytically in the synthesis of urea by the livers of other mammals, of a tortoise, and of a frog. Bird and snake livers, as would have been anticipated, form no urea when ammonia, ornithine and lactate are provided: uric acid is formed instead.

TABLE 19. ARGINASE CONTENTS OF VARIOUS
TISSUES (ARBITRARY UNITS)
(Data from Hunter and Dauphinee)

Species	Liver	Kidney	Heart	Muscle
Cat	1280	2.7	.	.
Rabbit	369	0.9	.	.
Hen	0	.	.	.
Pigeon	0	18.3	.	.
Mud-turtle	14.2	0	.	.
Dog-fish	319	31	109	2.2
Herring	181	7	8.8	0.9
Other teleosts	8-110	1-5	.	.

The case of the fishes (Table 19) calls for some special comment. Teleostean fishes are characteristically ammonotelic rather than ureotelic, although their livers contain arginase. The rest of the ornithine cycle mechanism is lacking, however, and no urea is produced when ornithine is provided, together with ammonia and lactate. The elasmobranchs, by contrast, are ureotelic and here, unlike other ureotelic organisms, arginase is present in considerable concentrations in practically every part of the body. As yet the effect of ornithine on the synthesis of urea seems not to have been studied in elasmobranch tissues. Presumably the

study of urea synthesis in such tissues is difficult since, to ensure survival of the tissues, it is necessary to work in a medium which resembles elasmobranch blood, which itself already contains a high proportion of urea. That arginase is present all over the elasmobranch body suggests that the synthesis of urea may not be confined to the liver, as it is in other ureoteles, and it is perhaps significant that, whereas hepatectomy in the mammal is followed by cessation of urea production, the hepatectomized dog-fish maintains its normal high level of uraemia.

As far as the invertebrates are concerned there is, as has been stated, little reason to believe that ureotelism has ever been exploited. Arginase, however, is present in many such organisms, usually in traces, but occasionally in concentrations as high as those in mammalian liver. One can only suppose that here, as among the teleostean fishes, the *tour de force* necessary for the acquisition of the rest of the ornithine cycle has not been achieved.

In confirmation of Krebs's hypothesis we may refer to the observations of Schoenheimer, who fed animals on a diet containing heavy ammonia. Arginine was subsequently isolated from the carcasses and found to contain heavy nitrogen (N^{15}). The isotopic nitrogen was confined to the amidine group, for on alkaline hydrolysis the ornithine formed was free from N^{15} , the whole of the heavy nitrogen being present in the urea. Moreover, the heavy nitrogen content of the arginine was practically equivalent to that of samples of urea isolated from the urine of the same animals.

The mechanisms of urea synthesis have also been studied by Trowell, who used perfused rat liver as his material. In this work it was found that while ornithine accelerates synthesis, citrulline produces only transitory effects, while the rate of synthesis from arginine was often less than that observed when ammonia and ornithine were added. Trowell came to the conclusion that Krebs's hypothesis is untenable, but Krebs himself has answered Trowell's objections in a manner that appears to be satisfactory. The chief critic of the ornithine cycle, however, has been Bach, who is of opinion that there must be alternative

mechanisms for the synthesis of urea. In his most recent experiments Bach has shown that urea synthesis continues unimpaired in the presence of concentrations of ornithine high enough to inhibit arginase almost completely, and concludes, therefore, that it must be possible for urea to be synthesized by some system which does not involve the participation of arginase. But, while Krebs's hypothesis remains the object of criticism, it has still to be shown that it is grossly incorrect. In the meantime there is little information and a good deal of disagreement regarding the nature of the alternative pathways of synthesis, if such are indeed assumed to exist.

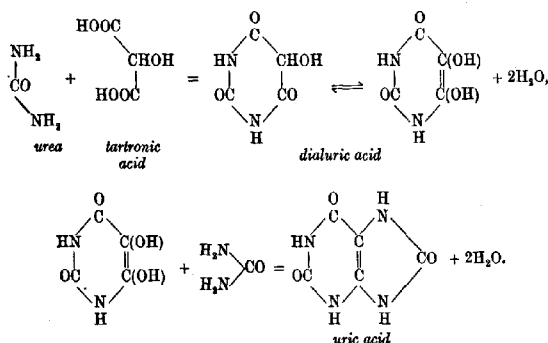
That the rate of synthesis of urea by liver tissue is accelerated by glutamine is undoubtedly true, and most of the speculations about alternative pathways of urea synthesis have regarded glutamine and glutamic acid as in some way involved. It might, however, be suggested that these substances do not actually participate in the synthesis but act, rather, as mechanisms for stabilizing the ammonia tension in the tissues and tissue preparations employed. To demonstrate the synthesis of urea by liver slices it is necessary to add ammonia; and ammonia, even in very low concentrations, is a powerful poison. In the presence of glutamine and glutamic acid, however, we may expect that, through the activity of the tissue glutaminase, the concentration of ammonia will be kept at a low level, corresponding approximately to that at which it is present in the intact liver *in situ*. The observed effects of glutamic acid and glutamine in surviving liver slices may well prove to be due to their action as 'buffers' which, by regulating the ammonia concentration, relieve the tissues of an ammonaemia which, in their absence, might be serious enough to result in an abnormally low rate of urea production.

SYNTHESIS OF THE END-PRODUCTS: URIC ACID

Uric acid is just as important an excretory product among uricotelic animals as is urea in those that are ureotelic, but very little is known about its mode of synthesis. Hepatectomy in the dog is followed by cessation of urea production, and hepatectomy

in the goose is similarly followed by cessation of the synthesis of uric acid. In both these cases hepatectomy leads to the accumulation of ammonia in the blood, and it follows that, just as urea is synthesized from ammonia in the liver of the dog, so too is uric acid synthesized from ammonia in that of the bird. Early experiments with perfused goose liver confirmed this conclusion, and showed convincingly that the uric acid excreted by birds is formed from ammonia.

The suggestion was made quite early that uric acid might be synthesized by way of urea, two molecules of the latter combining with one of some 3-carbon material to form the purine ring system, perhaps as follows:



Perfusion experiments were performed on goose liver in which urea was added to the perfusion medium and, in some cases, small yields of extra uric acid were observed, but a recent statistical analysis of the results has shown that the yields are not significant. Other experiments which gave results in favour of the hypothesis were carried out by feeding urea, together with 3-carbon compounds, to birds. In this case substantial yields of extra uric acid were recorded, but here, too, more recent work has shown that the results are not valid, for if urea is injected instead of being fed, there is no increase in the output of uric acid. The urea administered is quantitatively excreted in the

urine, and the earlier results must therefore be attributed to bacterial activity in the gut. Acting upon urea, the intestinal micro-organisms presumably liberate ammonia, which is absorbed and, as we know, can be converted into uric acid by the liver.

Quite apart from other considerations, it is known that the liver of birds and other uricotelic animals contains little or no arginase and, until it has been definitely established that urea can be synthesized through mechanisms not involving that enzyme, there seems little probability that urea could be produced in the avian liver, let alone be further converted into uric acid.

Numerous experiments have been performed by Clementi and his pupils, all of which contradict the view that urea is an intermediary in the synthesis. Liver perfusion and injection experiments alike have been used, urea being administered together with a large variety of 3-carbon compounds, and even with dialuric acid, but in no case has any significant synthesis of uric acid been obtained. Krebs, using liver slices, was able to confirm the old observation that ammonia can be built up into uric acid by bird liver, but he, like the others, could find no indication that any synthesis can take place from urea.

There is a hint that histidine and arginine may play some part in the synthesis of the purine ring system, for as Hopkins and Ackroyd showed a good many years ago, young rats stop excreting allantoin if deprived of these amino-acids. Allantoin is the normal end-product of purine metabolism in the rat, and is formed by the enzymatic oxidation of uric acid. The rat, however, is a ureotelic animal, and we cannot necessarily argue from this to the case of a purely uricotelic organism.

What little further knowledge we have was gained by taking advantage of the fact that, whereas the livers of most birds, e.g. the domestic hen and the goose, form uric acid from added ammonia, that of the pigeon does not yield uric acid but a precursor of some kind which, under the influence of enzymes present in the kidney, undergoes conversion into uric acid itself. Schuler and Reindel were the first to study this problem. Working with pigeon-liver slices they demonstrated that, whereas small amounts

of uric acid are formed by liver or kidney alone, much more is produced when both tissues are allowed to act simultaneously, or when the action of the liver is followed by that of the kidney. Krebs and his co-workers collected enough of the intermediary body for identification, and showed that it is hypoxanthine. Schuler and Reindel confirmed this result, inasmuch as they, too, isolated a purine body, which they believed to be xanthine, while subsequent repetition of the work by Krebs confirmed his original finding of hypoxanthine. The reason for this interesting dissociability of uricogenesis in the pigeon is that, whereas the livers of most birds contain xanthine oxidase, that of the pigeon does not. In this bird, xanthine oxidase is present in the kidney, and hypoxanthine formed in the liver is passed on to the kidney and there catalytically oxidized to uric acid, presumably by way of xanthine. At present, therefore, we are as far as ever from knowing how the purine-ring system is synthesized: all we can say for certain is that the starting material is ammonia and that hypoxanthine, and by presumption xanthine also, are intermediaries in the synthesis.

Little work has been done on uricogenesis in the reptiles. As far as the uricotelic invertebrates are concerned, a good deal of work has been done, but the results are, at best, inconclusive. The outlook in this field has been much prejudiced by the view, now abandoned in so far as it affects uricotelic vertebrates, that urea is an intermediary in the synthesis. The best attitude to adopt at the present time is one of ignorance.

SYNTHESIS OF OTHER END-PRODUCTS:

TRIMETHYLAMINE OXIDE

The excretion in some cases of as much as 50% of the total nitrogen in the form of trimethylamine oxide by marine fishes suggests that this substance must have a synthetic origin in these fishes. It is possible that a part at least may originate as such in the food. Many marine organisms, including the small Crustacea which form an important article of diet for many fishes, contain substantial amounts of trimethylamine oxide which, it

may be thought, is taken over by the feeder and excreted unchanged. Another possible source is glycine betaine. This substance occurs abundantly in some animals and in many plants. It is known that if cows are fed on sugar-beet residues, which are a rather rich source of glycine betaine, trimethylamine oxide appears in the milk. But although betaine gives rise to trimethylamine oxide when administered to cows, it is generally believed that the conversion is not due to the tissues of the cow itself, but rather to the activities of the symbiotic micro-organisms which inhabit its rumen. Animal tissues do not, in general, appear to be capable of converting betaine into trimethylamine oxide, though trimethylamine itself is oxidized by mammalian tissues to yield the oxide. One can do little more at present than guess at the extent to which such a conversion can take place in fishes, and in any case there remains to be explained the excretion of trimethylamine oxide by marine, yet not by fresh-water, fishes, for there is no reason to think that the food of fresh-water species contains less glycine betaine than that of marine forms.

It is conceivable that trimethylamine itself might be formed by the biological methylation of ammonia, and subsequently oxidized to the oxide in animal tissues; mechanisms for methylation are known (p. 163), and it is known too that trimethylamine can be oxidized to its oxide in the tissues of some animals.

Guanine (2-amino-6-oxypurine). Special mention must be made of guanine, for this purine appears to replace uric acid in the excreta of spiders. In spite of the popular belief to the contrary, the spiders constitute a group that is morphologically quite distinct from the insects: they demonstrate their independence chemically, too, by excreting guanine in place of uric acid. *Guanine, if anything, is even less soluble than uric acid*, and contains one amino-group per molecule over and above the four ring-bound nitrogen atoms of the purine ring. Evidently, therefore, guanine is well qualified to take over the excretory functions of uric acid.

The manner of its synthesis is unknown: conceivably it might arise from xanthine by amination, but there is no experimental evidence whatever on this point.

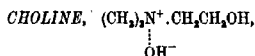
CHAPTER XI

SOME SPECIAL ASPECTS OF NITROGEN METABOLISM

NITROGENOUS substances of many different kinds have been isolated from time to time from various animal materials, and although their origins, functions and metabolic fates are known in only a few cases, some mention of these compounds and their distribution seems desirable here. We shall consider first a group of compounds chemically related to choline, then a group of betaines, a rather large group of derived guanidines, and some iminazole bases.

DISTRIBUTION OF CHOLINE, TETRAMETHYL AMMONIUM HYDROXIDE, TRIMETHYLAMINE AND TRIMETHYLAMINE OXIDE

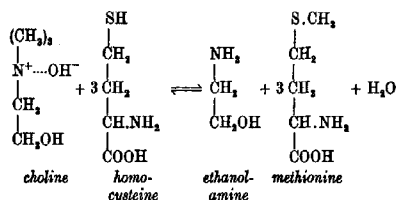
Many different animal tissues have been found to contain larger or smaller amounts of highly methylated nitrogenous compounds such as trimethylamine oxide and various betaines, and the suggestion has often been made that they must arise from choline. At the present time, however, it seems less probable that they arise directly from choline than that they are formed by the methylation of other substances, choline entering into the picture only as a source of methyl groups.



is probably universally distributed as the basic constituent of phospholipoids of the lecithin type. Traces of free choline have been isolated from animal tissues of many kinds, but it is doubtful whether it occurs in the free state to any great extent. Probably it arises by autolysis, for, to take only one example, dog liver worked up as rapidly as possible after the death of the

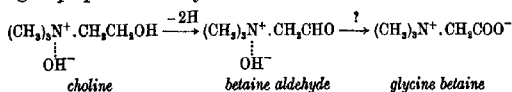
animal contains only 0-43 mg. free choline per kg., rising to 136-164 mg. per kg. if the tissue is allowed to stand for 5 hr. before being worked up.

In addition to its importance as a constituent of the lecithins, choline is important as the raw material from which acetylcholine, the neuro-hormone of the parasympathetic nervous system, is presumably synthesized. In comparatively recent times it has been discovered that the methyl groups of choline can be transferred to homocysteine to yield methionine which, in its turn, acts as a biological methylating agent (see p. 236):



The other product, ethanolamine (cholamine), it will be remembered, replaces choline in phospholipoids of the cephalin type.

The metabolic importance of choline may be judged from the fact that it appears to be an essential dietary constituent, usually classified with the B₂ group of vitamins: it is perhaps essential as a source of —CH₃ groups. Little is known about its metabolism however. The discovery of a choline dehydrogenase in mammalian liver indicates that choline can be oxidized, the product being betaine aldehyde, and there is a possibility that the latter may then be further oxidized to betaine itself, e.g. by the group-specific aldehyde oxidase of the liver:



TETRAMETHYLAMMONIUM HYDROXIDE ('tetramine'), (CH₃)₄N⁺...OH⁻, has been isolated from only one animal source, the coelenterate *Actinia equina*, from which it was obtained in remarkably high yield, some 12 g. of the chloride being obtained from 33 kg. of

the fresh material. Like most quaternary ammonium bases it has a powerful paralytic action and may, it is thought, be responsible for the paralytic 'sting' which many coelenterates are capable of inflicting.

TRIMETHYLAMINE, $(\text{CH}_3)_3\text{N}$, and
TRIMETHYLAMINE OXIDE, $(\text{CH}_3)_3\text{N}\rightarrow\text{O}$,

have been obtained from many animal sources, vertebrate and invertebrate alike. As a rule the oxide occurs in quantities that completely overshadow those of the free base and, in general, it seems that trimethylamine arises from its oxide through bacterial action. The characteristic odour of dead marine fishes, which incidentally is not observable in fresh-water species, is largely due to free trimethylamine formed by the action of putrefactive bacteria upon trimethylamine oxide present in the tissues. The isolation of free trimethylamine from fish muscle has been reported by several workers, but it seems highly probable that such results are attributable to the use of stale material. Perfectly fresh fish muscle contains traces at most of the free base.

Free trimethylamine has long been known as a trace constituent of mammalian urine, and it has been detected also in human menstrual blood. Little is known about its metabolic origin and fate except that it is almost quantitatively converted into its oxide when fed to mammals (cow, man).

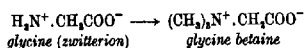
Perhaps the most striking fact that has emerged in connexion with trimethylamine oxide is that, while it is present in a great variety of marine animals, it seems never to occur on a substantial scale among fresh-water organisms. Its function in the fishes has been studied a good deal. That it occurs only in marine species suggests that it might play a part, analogous to that of urea in marine elasmobranchs, in the regulation of the osmotic pressure of the blood, for it resembles urea in being nearly neutral and relatively innocuous. Elasmobranch bloods contain about 100-120 mm per litre, as compared with about 330-440 mm of urea. It accounts therefore for 20-25% of that part of the total osmotic pressure not due to salts. Further, the

concentration of trimethylamine oxide in the urine of marine elasmobranchs is only about one-tenth as great as that present in the blood, so that this substance, like urea, is actively retained by the elasmobranch kidney. Probably, therefore, it plays a significant part in osmotic regulation in these fishes.

Among teleosts, however, matters are different. The trimethylamine oxide content of fresh cod muscle, for example, has been estimated at about 20 mm per kg., i.e. only about one-fifth of the amount present in elasmobranch muscle. Furthermore, there is no reason to believe that the substance is retained, for it is one of the major nitrogenous constituents of the excreta of marine teleosts. It is therefore unlikely that trimethylamine oxide plays any significant osmotic role in marine teleosts; it is far more probable that it is essentially an excretory product and represents a detoxicated form of ammonia (p. 256). Its origin has been discussed in an earlier section (p. 272).

DISTRIBUTION OF BETAINES

Betaines of various kinds are widely distributed among animals and plants alike, but their mode of formation is still somewhat obscure. It has usually been supposed that they are formed by the methylation of simpler substances, glycine betaine arising from glycine for example:



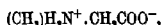
In cases in which a given betaine is known as a constituent both of plant and animal materials it is, of course, always possible that the animal derives the substance from plant food, either directly or indirectly, according as it is herbivorous or carnivorous. Several of the known betaines, however, appear to be peculiar to animal tissues.

GLYCINE BETAINE

This is very widely distributed in plants and has been found in quantities of the order of 0.2% in the muscles of many invertebrates. Little is known about its origin or function unless,

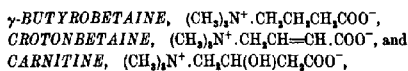
indeed, it arises from plant foods. There is a little evidence that it can be formed in mammals by the oxidation of choline (p. 275).

As a rule, large amounts of the betaine are found in association with small quantities of free glycine and vice versa, suggesting that the two are metabolically interconvertible. In one case, at least, glycine has been found side by side with sarcosine,

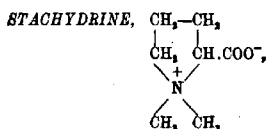


suggesting that the methylation of glycine is a step-wise process. That glycine and its betaine are interconverted by an exchange of methyl radicals seems very probable in view of the recent discovery that the methyl groups of glycine betaine can be transferred, like those of choline, to homocysteine.

If glycine betaine is fed to cats, dogs or rabbits it is largely recoverable from the urine. Traces of trimethylamine are found at the same time, but this, according to prevailing opinion, is formed by bacterial intervention.

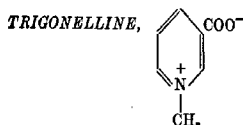


are closely related one to another, and all three are in part interconvertible, as has been shown by subcutaneous administration to dogs. Their origins, functions and fates are altogether obscure. Unlike glycine betaine, these substances are not known to occur in plant tissues and are therefore supposed to arise *de novo* in animals. It seems improbable that γ -butyrobetaine can arise by methylation from γ -aminobutyric acid, since the latter has not been found in animal materials.

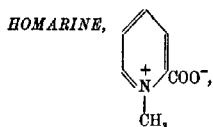


a cyclic compound, has been isolated only from one animal source, the Noah's Ark mussel, *Arca noae*, an animal that contains a remarkably rich collection of nitrogenous compounds.

Stachydrine is well known as a constituent of plant materials, and, since *Arca noae* is herbivorous, we must suppose until more information is forthcoming that its presence in this animal is dietary in origin.



has been isolated from mammalian urine. It arises to some extent, no doubt, in plant foodstuffs, but can arise also as an end-product of the metabolism of a member of the B₂ group of vitamins, viz. nicotinic acid. If large doses of this substance are given to mammals, a part is excreted unchanged, a part is conjugated with glycine to form nicotinuric acid, and a part undergoes *N*-methylation and gives trigonelline. This appears to be but one instance of a rather general phenomenon, for it has been known for many years that the mammalian organism is able to accomplish *N*-methylation of the pyridine ring, for pyridine itself suffers this fate.

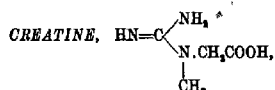


appears to be peculiar to animals, and has been found in a few invertebrates, including *Arca noae*. It is a methylated picolinic acid, isomeric with trigonelline, and may conceivably arise by biological methylation of picolinic acid, though the latter has not so far been discovered as a biological product.

DISTRIBUTION OF BASES RELATED TO GUANIDINE

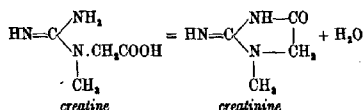
The predominant members of this group are arginine and creatine. With certain notable exceptions to which we shall refer later it may be said that free creatine is found only among vertebrates,

and free arginine only among invertebrates. These bases are mainly confined to muscular tissues, in which they are present in the form of their very labile phosphates, the phosphagens (p. 333).



is an amino-acid, though not of the usual α -amino-type, nor does it occur in proteins. The fact that it replaces arginine in the muscles of vertebrates led to the belief that it must be formed metabolically from arginine, a view upheld by the common presence of an amidine radical in both substances.

Creatine itself does not usually appear in mammalian urine, but undergoes slow, spontaneous conversion under physiological conditions of temperature and pH into the internal anhydride, *creatinine*,

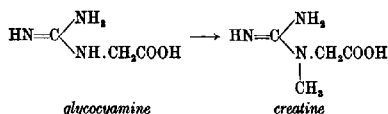


Creatinine is a normal excretory product among mammals, and its origin in creatine has been demonstrated by feeding experiments in which creatine, 'labelled' with heavy nitrogen, was administered. When heavy nitrogen was introduced into the amidine group, the isotope was recoverable in the same position in the amidine residue of creatinine isolated from the urine.

If small doses of creatine are administered to normal animals the substance is temporarily accommodated in the muscles, as may be demonstrated by comparing the muscles of animals thus fed with those of controls. Later, increased outputs of creatinine are observed. Larger doses of creatine give rise to a definite creatinuria, however, together with an increased excretion of creatinine. In certain pathological conditions of the muscles, the muscular dystrophies, which are characterized by extensive wasting of the muscular tissues, creatine is still produced in the organism at the usual rate, but, as the muscles are no longer

able to accommodate it, it is excreted in the urine. Creatine is also excreted after the amputation of a limb. Evidently, therefore, creatine is made elsewhere than in the muscles, and is excreted if the muscles cannot make use of it. Animals suffering from muscular dystrophy, or from which some major muscle masses have been removed, therefore play an important part in studies of creatine formation.

For many years it was believed that creatine must arise from arginine, for reasons that we have already mentioned. Little evidence could be found in favour of this hypothesis, but it was discovered that glycocyamine, a compound which is intermediate in structure between arginine and creatine, gives rise to creatine in feeding experiments. Glycocyamine requires only to be biologically methylated to yield creatine:

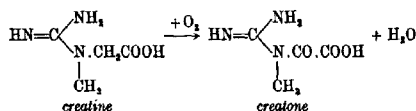


Many other nitrogenous substances have been tested in a search for precursors of creatine, and it has been shown that several amino-acids can lead to some creatine formation. Outstanding among these compounds is glycine, which yields creatine more freely than does arginine. In recent years it has been found that if kidney slices are allowed to act simultaneously upon glycine and arginine, glycocyamine is formed. By the subsequent action of liver tissue upon glycocyamine in the presence of methionine, creatine is produced. The synthesis of creatine therefore involves three amino-acids: first glycine is transaminated at the expense of arginine and yields glycocyamine, and the latter is then trans-methylated at the expense of methionine (for equations see p. 163).

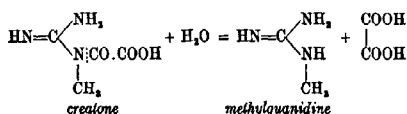
The correctness of this scheme has been demonstrated in feeding experiments involving the use of isotopic 'markers'. If arginine containing N^{15} in its amidine group is administered to animals, the heavy nitrogen can be recovered from the amidine radical of creatine isolated from the muscles. Similarly, if glycine

containing N^{15} is fed, heavy nitrogen is again recoverable from creatine isolated from the muscles but not, this time, in the amidine radical. Similarly, the administration of trideutero-methionine, i.e. methionine of which the methyl group contains heavy hydrogen, gives rise to a creatine which also carries deuterium in its methyl group.

Nearly related to creatine is another base, known as *creatone*. This substance has been isolated from many vertebrate materials but not from invertebrate sources. It is an artefact derived from creatine during the processes of isolation. The methods used in working up the nitrogenous bases of biological materials usually involve the use of mercuric salts as precipitants, and it has been shown that, in the presence of mercuric compounds, creatine readily undergoes atmospheric oxidation to creatone:



Creatone itself is readily hydrolysed to give oxalic acid and another artefact, *methylguanidine*:



Methylguanidine, like creatine and creatone, has been isolated from numerous vertebrate muscles but not, so far at any rate, from any invertebrate sources. *Guanidine* itself has been obtained from a few animal sources, and may perhaps arise by the further degradation of creatine, arginine, or some other guanidine derivatives.

ARGININE

This is a relatively common amino-acid and is present, probably, in all proteins. Quite apart from the special part it plays in the muscle metabolism of invertebrates, arginine enters into the composition of the tissue proteins of animals of every kind. It

will be recalled that the rate at which arginine can be synthesized in young rats and chicks appears to be limited and can act as a limiting factor upon their growth-rate. Whether invertebrates are less or more adept at the synthetic production of arginine we do not know: for present purposes we may reasonably assume that it is normally forthcoming in sufficient quantities in the diet.

Distribution of the Phosphagens. Although arginine has been detected in, and in many cases actually isolated from, the tissues of representative members of almost every phylum and class among the invertebrates, there is no reason to believe that it occurs in vertebrates except, of course, as a constituent of proteins, and also, in small concentrations, as a transitory metabolic intermediate.

Just as non-protein arginine is confined to members of the invertebrate phyla, so too is creatine confined to the vertebrates. Arginine and creatine alike occur mainly in the muscles, and in the form of their phosphates, the phosphagens; the following discussion refers to these latter substances and the free guanidine bases arising by their decomposition. Creatine has been isolated from representative members of every vertebrate group but never, except occasionally and in traces, from invertebrates. To these generalities, however, there are a few noteworthy exceptions, as is shown in Table 20. Of all the invertebrate types studied, the Echinodermata are exceptional in that they alone include species in which the presence of creatine has been satisfactorily demonstrated. In the echinoids (sea-urchins) arginine and creatine occur side by side, while in the ophiuroids (brittle-stars) only creatine could be detected.

No comparable cases have been found in the Vertebrata proper, but among the Protochordata, a group of creatures which resemble the true vertebrates in their possession of a primitive notochord, one similar case has been recorded. The Protochordata comprise three groups, the Tunicata (sea-squirrels), the Enteropneusta (a small group of worm-like animals) and the Cephalochorda (lancelets). These Protochordata are of special interest, since they are regarded on morphological grounds as lying on

the border-line between the true Vertebrata on the one hand and the invertebrate phyla on the other.

Of the Cephalochorda it may be said that they resemble the vertebrates rather than the invertebrates, for not only do they

TABLE 20. DISTRIBUTION OF ARGININE AND CREATINE
IN THE ANIMAL KINGDOM

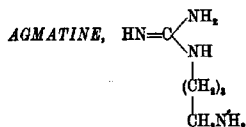
Phylum	Class	Arginine	Creatine
Protozoa		-	-
Porifera		-	-
Coelenterata	Scyphozoa	+	-
	Actinozoa	+	-
	Ctenophora	+	-
Platyhelminthes		+	-
Nemertea		+	-
Mollusca	Amphineura	+	-
	Lamellibranchiata	+	-
	Gastropoda	+	-
	Cephalopoda	+	-
Annelida	Polychaeta	+	-
	Oligochaeta	+	-
Gephyrea		+	-
Phoronidea		+	-
Arthropoda	Crustacea	+	-
	Insecta	+	-
	Arachnida	+	-
Echinodermata	Crinoidea	+	-
	Asteroidea	+	-
	Ophiuroidea	-	+
	Holothuroidea	+	-
	Echinoidea	+	+
Protochordata	Tunicata	+	-
	Enteropneusta	+	+
	Cephalochorda	-	+
Vertebrata	Fishes	-	+
	Amphibia	-	+
	Reptilia	-	+
	Aves	-	+
	Mammalia	-	+

possess a well-developed notochord but, chemically speaking, the relationship is clear from the fact that they contain creatine but not arginine. The Tunicata resemble the invertebrates rather than the vertebrates, for, quite apart from their general appearance and mode of life, their tissues contain arginine but not, so far as we know, creatine. In the Enteropneusta, however, we

find a case in which there is evidence for the presence of arginine and creatine side by side in the musculature.

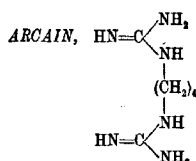
The Enteropneusta thus appear to be related to the Vertebrata in that they contain creatine and, at the same time, to the invertebrate phyla in that they contain arginine. In particular, they show an affinity with the Echinodermata, for only in these two groups have arginine and creatine been found to co-exist in significant quantities. Similar relationships were established on purely morphological grounds by the classical investigations of Müller, Metschnikow and Bateson. As Bateson showed, the Enteropneusta possess, in addition to their gill-slits, a short but well-defined notochord, both of which establish their relationship to the Vertebrata. But in their larval forms the Enteropneusta so closely resemble the echinoderms that their larvae were classified with those of the Echinodermata before the adult forms were discovered. It was Metschnikow who, some time later, showed that the *Tornaria* larva, far from being an echinoderm larva as was previously believed, gives rise in the adult form to *Balanoglossus*, an enteropneust.

From the phylogenetic standpoint the natural distribution of these two bases, arginine and creatine, is clearly of considerable interest. The chemical evidence available favours Bateson's views on the origin of vertebrates by pointing to the echinoderms and the enteropneusts as links between the invertebrates on the one hand and the vertebrates on the other. Other theories of vertebrate descent have been put forward by morphologists in the past, but for none of them has any substantial chemical support yet been found.



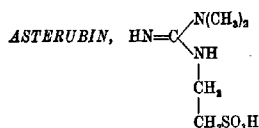
is a strong base which can be prepared chemically by the decarboxylation of arginine. It has been isolated from a few invertebrate sources, notably from the sponge, *Geodia gigas*, and from several cephalopods. It is said to be present in the poisonous

The discovery of octopine has been of particular interest on account of its structural relationship to the hypothetical intermediary involved in transamination and transdeamination (p. 161).



(tetramethylene diguanidine), has been isolated only from *Arca noae*. It was again isolated from a second batch of the same organism, but none could be found in another lamellibranch, *Mytilus edulis*. Structurally it is nearly related to the synthetic substance synthalin (decamethylene diguanidine), which has a profound effect in lowering the level of the blood sugar in mammals, and has been used experimentally for that purpose in the treatment of diabetes mellitus.

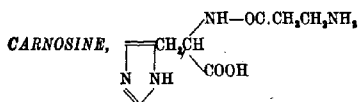
How arcain is formed we do not know. Several suggestions have been made, but there is no evidence to favour one rather than another. Probably it must arise in some way from arginine.



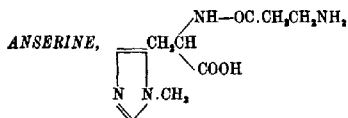
is not, as its name might suggest, a pigment, but a sulphur-containing guanidine derivative which, up to the present, has been obtained only from two species of star-fishes. Its mode of synthesis is unknown. It may be considered as being derived from an amidinated taurine, taurine itself being widely distributed in nature. Asterubin, like taurine itself, belongs to the class of sulphonic acids, a somewhat rare group in nature.

DISTRIBUTION OF IMINAZOLE BASES

In addition to the amino-acid histidine, in which the iminazole ring is present, several other iminazole compounds have been found in animal tissues. Of these the most widely distributed are carnosine and anserine, which appear to be peculiar to the muscular tissues of vertebrates, for neither has ever been found among the extractives of invertebrate materials.



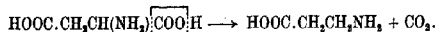
(β -alanylhistidine), is something of a biological curiosity, being derived from the rare β -amino-acid, β -alanine. It is, therefore, not a typical dipeptide, since all such are derived wholly from α -amino-acids. Carnosine has been known since 1900, when it was isolated from Liebig's meat extract, and since that time it has been obtained from the muscles of representatives of all classes of vertebrate animals.



a methylated carnosine, is also a derivative of β -alanine. It was first isolated from the muscle of the goose, *Anser*, from which it received its name. It was thought for a time that carnosine and anserine mutually replace each other, but in more recent times it has become evident that the two not uncommonly occur side by side in one and the same tissue.

The function of these peculiar bases is very obscure. Attempts have been made to show that the amount of these iminazole bases is correlated with the activity of the muscle, but without success. Indeed, the only hint we have about their possible function is the fact that both are strong buffers.

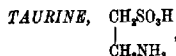
How carnosine and anserine are formed in animal tissues is still unknown. Anserine may perhaps arise by the transmethylation of carnosine. Carnosine itself can replace the essential amino-acid histidine in the diet of rats, from which we may conclude that carnosine can be hydrolysed in the tissues. It is possible, therefore, that it may arise by condensation between β -alanine, or some forerunner of β -alanine, and histidine. If this is so, the origin of β -alanine remains to be accounted for. Certain bacteria can produce it by the decarboxylation of aspartic acid, and it may be that a similar process can take place in plants:



There is no indication, however, that this reaction takes place in animal tissues, but, if it occurs in plants, β -alanine is presumably available to animals in their food.

DISTRIBUTION OF OTHER NITROGENOUS COMPOUNDS

Many nitrogenous substances have been isolated at one time or another from animal tissues of various kinds, including free amino-acids (notably glycine) and purine bases (p. 301), in addition to those already discussed. Of these, taurine deserves special mention, if only because it occurs in very large amounts in certain tissues.



has long been known as a constituent of the bile of vertebrates, in which it is present in conjugation with cholic and other bile acids (p. 201). Its metabolic relationships with cysteine have already been discussed (p. 234).

That free taurine occurs, often in very notable quantities, among invertebrates has been known for many years. Thus the adductor muscle of the edible mussel (*Mytilus edulis*) contains 4-5%, while the muscles of the annelid worm, *Audouinia spiranthis*, contain 3% of taurine. Muscles of vertebrate organisms also contain free taurine, though in much smaller amounts. Its function, however, is unknown: conceivably it plays an important part in regulating the osmotic pressure of the contents of those cells in which it is present in high concentrations.

CHAPTER XII

METABOLISM OF PURINE DERIVATIVES

NUCLEOPROTEINS

IN this chapter we have to deal with a group of natural compounds called nucleoproteins and with certain products arising by their breakdown. Nucleoproteins contribute only a small proportion to the total nitrogen of any average diet and account for only a small fraction of the total nitrogen of most cells and tissues but, as their name implies, they occur especially in cell nuclei, and are very important substances. Any cell that contains a high proportion of nuclear material will form a good source of nucleoproteins, and glandular materials such as pancreas and thymus have been used extensively for their preparation. The richest source of all seems to be the heads of ripe spermatozoa: indeed, it has been estimated that the nucleoprotein content of fresh spermatozoa is from 50-80 % of the total solid matter. Ripe fish roes, therefore, are a valuable source of nucleoproteins and their derivatives.

It is only during the last few years that the nucleoproteins have attracted very much interest. The early preparations were rather crude, insoluble substances, unattractive to the pure chemist, and the present phase of interest began when it was discovered that certain plant viruses can be isolated in crystalline form and are, in fact, nucleoproteins. Unlike the products formerly obtained, these virus proteins give beautiful, water-soluble crystals. A number of plant viruses have now been shown to consist of crystallizable nucleoproteins, and among animal viruses at least one, vaccinia, the virus of cowpox, contains, even if it does not consist entirely of, a nucleoprotein.

It had hitherto been supposed that viruses are living organisms, small enough to pass through the pores of bacterial filters and to be invisible under the microscope. Yet virus diseases can be transmitted to healthy plants either by inoculation with sap from an infected plant or with the crystalline virus protein. If

healthy plants are infected by means of a pure virus nucleoprotein and allowed to develop the disease, the virus protein can later be isolated in quantities very much in excess of those used for the original inoculation. These discoveries produced some shocks among biologists who, as a whole, had always supposed that there exists some sharp line of demarcation between things that are living and things that are not. Yet, in these virus proteins, we have something which can be crystallized and can at the same time transmit disease from a sick to a healthy plant and, moreover, reduplicates or 'reproduces' as the disease develops.

More recently still it has been shown that chromosomes, too, consist largely of nucleoprotein material, and that we must look on the nucleoproteins not only as the causative agents of a large number of infectious diseases, but also as the vehicles whereby hereditary characteristics are transmitted from parents to offspring. Virus proteins and chromosomes have several important features in common. Both can exist as long, fibrous structures, and both, given the interior of the right kind of cell as environment, can reduplicate. Further, both show the phenomenon of mutation, either in the course of nature or under the artificial influence of X-rays or γ -radiation. Just as new strains of organisms can suddenly appear as a result of genetic mutations, so, too, new virus strains can suddenly appear as a result of mutations in the old. This process of mutation among viruses is quite probably the reason for the hitherto inexplicable variability in the virulence of many virus-borne diseases, e.g. influenza.

It will be clear to the reader that the nucleoproteins are at present a subject of manifold interest. A full knowledge of their chemical constitution must necessarily await further developments in protein chemistry as far as their protein components are concerned, but, in the meantime, considerable strides are being made in the chemistry of their non-protein, prosthetic components, the nucleic acids.

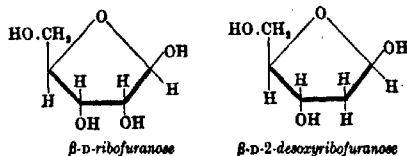
NUCLEIC ACIDS

Nucleoproteins are conjugated proteins, formed by the essentially salt-like union of a nucleic acid with a basic protein such as a protamine or a histone. If thymus gland material is

macerated with large volumes of water the thymus nucleoprotein is extracted, and the protein component, in this case a histone, can be precipitated by saturation of the extract with sodium chloride. On the further addition of ethyl alcohol, nucleic acid is precipitated as a fibrous mass.

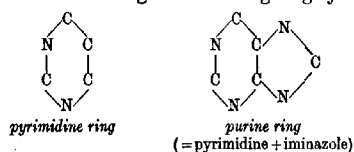
Two chief types of nucleic acid have so far been recognized, one obtained from thymus gland and the other from yeast. These acids are built up from smaller units known as nucleotides, which yield on hydrolysis one molecule each of a nitrogenous base, a pentose sugar and phosphoric acid. They are, in fact, phosphate esters of the *N*-glycosides of certain nitrogenous bases. According to the older work, four of these nucleotide units go to make up one molecule of nucleic acid, but recent determinations of the particle weight, carried out by the method of ultracentrifugation, give values ranging from 200,000 to 1,000,000. Physical studies show that the particles are long, rod-like objects, each of which appears to consist of aggregates of smaller units. These aggregates can be made to depolymerize in various ways, and the smallest units so far obtained by disaggregation have particle weights of the order of 15,000. If, as seems possible, these are really molecular units, it follows that each molecule of nucleic acid must contain many more than the four nucleotide radicals formerly postulated. At present the tendency is to think of nucleic acids as being built up by the union of large numbers of nucleotide units, much in the way that proteins are built up by the union of large numbers of amino-acid units.

The most striking differences between nucleic acids of the yeast and thymus types lie in the nature of the sugar radicals involved in the nucleotide units. Nucleic acid prepared from yeast contains β -D-ribofuranose, while that from the thymus gland of animals contains β -D-2-desoxyribofuranose:



It was at one time believed that plant and animal cells always and only contain ribonucleic and deoxyribonucleic acids respectively. This view has turned out to be entirely erroneous. Probably all cells contain nucleic acids of both types, the desoxy-ribose compound preponderating in the nucleus and the ribose compound in the cytoplasm generally.

Both types of nucleic acids contain phosphate radicals, and both contain bases belonging to the purine and pyrimidine groups, i.e. bases containing the following ring systems:



Hydrolysis of the nucleic acids by means of dilute acids or the appropriate enzymes yields the following recognized products:

	CYTOPLASM	NUCLEUS
	RIBONUCLEIC ACID (<i>'yeast nucleic acid'</i>)	DESOXYRIBONUCLEIC ACID (<i>'thymonucleic acid'</i>)
<i>Pentose:</i>	D-ribose	D-desoxyribose
<i>Pyrimidines:</i>	cytosine; uracil	cytosine; thymine
<i>Purines:</i>	adenine; guanine	adenine; guanine

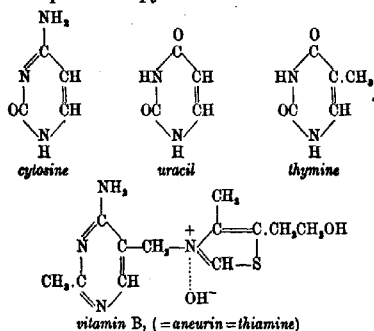
The chief differences lie, therefore, in the nature of the pentose radicals, and in the presence of thymine and of uracil in desoxy-ribonucleic and ribonucleic acids respectively. Since at least these four different bases are found in whichever nucleic acid is taken, it follows that at least four nucleotide radicals enter into the composition of the nucleic acid concerned; but, as has been pointed out, there is reason to believe that many more than four nucleotides, perhaps some fairly high multiple of four, are present.

DIGESTION OF NUCLEOPROTEINS

The salt-like union between the nucleic acid and the basic protein component of a typical nucleoprotein is disrupted by the acidic contents of the stomach, the protein fragment being digested along with the other food proteins. Nucleic acid is further split by enzymes contributed by the pancreatic and

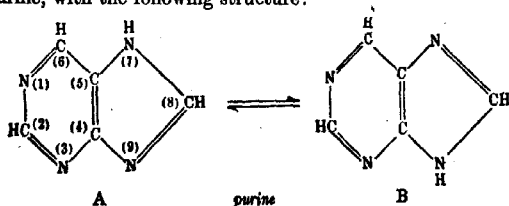
intestinal juices. Our knowledge of these enzymes is somewhat fragmentary at present, but they appear to comprise (i) a *nuclease*, which liberates the component nucleotides (p. 85), (ii) a *nucleotidase*, which catalyses the dephosphorylation of the nucleotides, yielding nucleosides, which are glycosides of the nitrogenous bases (p. 85), and (iii) a *nucleosidase*, which hydrolyses the nucleosides to liberate the basic and glycosidic components.

Relatively little is known about the fates of the pyrimidine bases and we shall not consider them here. Their structures are appended as a matter of interest, and in passing it should be noticed that a large and important group of drugs, the barbiturates, are structurally related to the pyrimidine group, and that vitamin B₁ also is a pyrimidine derivative:



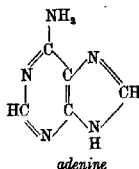
Similarly, it may be pointed out, caffeine and a number of other important drugs are methylated purines.

Considerably more is known about the fates and functions of the purine bases. The parent of this group of compounds is purine, with the following structure:



The iminazole portion of the system is tautomeric, and it should be noticed that while form A is the one usually figured in textbooks, B is probably the more biologically important, for when the purines enter into glycoside formation with ribose the union takes place at position (9) according to all available evidence (see structure of adenylic acid, below).

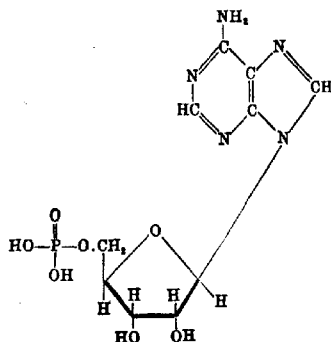
The most important of the purine bases is *adenine*, 6-amino-purine:



NUCLEOSIDES AND NUCLEOTIDES

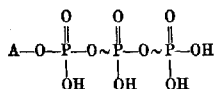
Adenosine (adenine-9- β -D-ribofuranoside) is perhaps the most important member of the family of nucleosides, since by phosphorylation in position (5) of the ribose radical it gives rise to the nucleotide, adenylic acid.

Adenylic acid, also known as *adenosine monophosphate* (AMP), has the following structural formula, according to all the available evidence:



Further phosphate radicals can be attached to the first to yield *adenosine diphosphate* (ADP) and *adenosine triphosphate* (ATP). These substances play a fundamental part in the energetics of living systems.

The molecule of adenosine triphosphate contains two energy-rich bonds, i.e. bonds the hydrolytic rupture of which yields about 12,000 cal. per g.mol. of phosphate liberated, as compared with the 2000–3000 cal. or thereabouts which are liberated by the hydrolytic fission of the 'ordinary', energy-poor bonds of phosphate esters in general (see Table 21). In ATP itself the three phosphate radicals are believed to be attached end to end, so that, if we represent adenosine by A and the energy-rich bonds by the symbol ~, the structure of ATP can be represented as follows:



The special importance of ATP arises from the fact that, under the influence of the appropriate enzymes, its terminal phosphate radical, *together with the energy of the terminal energy-rich bond*, can be transferred intact to other substances, so that energy is, as it were, forced into the phosphate receptor. This appears to be a fundamental operation in the synthesis of complex biological compounds from simpler starting-materials, and as far as we know at the present time, synthetic operations of this kind can only be accomplished at the expense of the terminal energy-rich bond of adenosine triphosphate. ATP itself can be reformed from ADP at the expense of the free energy of certain metabolic processes that lead to the generation of new energy-rich bonds, but the transmission of these new bonds to other substances can only be accomplished through the intermediation of ATP.

Adenosine triphosphate can be split in any one of several ways in living cells. It may yield up one phosphate radical and one energy-rich bond in a transfer reaction of the kind just described (see p. 162 for examples), or, under the influence of an adenosine

triphosphatase, it may undergo hydrolysis to yield adenosine diphosphate, together with free, inorganic phosphate. In this latter case, however, the energy of the energy-rich bond is not conserved, as it is in the transfer reactions, but is set free. If the reaction is allowed to take place in a test-tube or flask the

TABLE 21. ENERGY-YIELDS OF HYDROLYSIS
OF PHOSPHATE BONDS
(After Lipmann)

Type and identity of bond	Heat of hydrolysis, (cal.) $-\Delta H$	Decrease in free energy (cal.) $-\Delta F$
Esters:		
$\begin{array}{c} \diagup \\ \text{C}-\text{O}-\text{P} \\ \diagdown \end{array}$ Glycerol- α -phosphate		2,350
Carbonyl phosphates:		
$\begin{array}{c} \diagup \\ \text{C}-\text{O} \sim \text{P} \\ \diagdown \end{array}$ Phosphoacetic acid	8,000	10,000
Phospho-enol-pyruvic acid	8,450	11,250
1:3-Diphosphoglyceric acid	8,250	11,250
Guanidine phosphates:		
$\begin{array}{c} \text{NH} \sim \text{P} \\ \diagup \\ \text{HN}=\text{C} \\ \diagdown \\ \text{NH} \end{array}$ Creatine phosphate	10,700	c. 10,000
Arginine phosphate	7,700	c. 10,000
Polyphosphates:		
$\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ -\text{P}-\text{O} \sim \text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$ ATP, ADP (per energy-rich bond)	12,000	c. 10,000

Note that the free energies of all the energy-rich phosphate bonds are approximately equal.

energy appears in the form of heat, but in an intact muscle cell it appears instead in the form of the mechanical energy of the concomitant muscular contraction. It is probably not without significance, therefore, that the adenosine triphosphatase of muscle is so intimately associated with the contractile protein of muscle, myosin, that even a partial separation of the two has not yet been accomplished.

In the electric organs of certain fishes (*Torpedo*, *Raia* sp., *Malapterurus*) an adenosine triphosphatase of a different kind

is apparently present, for myosin is lacking in the most highly developed of these organs, although they arise by the morphological transformation of embryonic pre-muscular cells. The electrical energy dissipated when such an organ discharges arises, according to present knowledge, from ATP once again.

Now energy-rich bonds arise in the course of numerous katabolic processes, especially in reactions involving dehydrogenation, and supplies of ATP are maintained in a metabolically active tissue by the transference of these newly formed bonds to ADP left after the previous breakdown of the triphosphate. In certain tissues, energy-rich bonds are 'stored' in the form of the phosphagens, the energy-rich phosphate derivatives of creatine and arginine respectively. These stored bonds cannot be used directly, but only through the ADP/ATP system. They can, however, be transferred directly to ADP, and the transfer is freely reversible. When such a tissue enters into physiological activity ATP is broken down and provides energy in the form characteristic of the particular organ. Unless or until new bonds are generated rapidly enough to resynthesize ATP as fast as it is used up, the tissue can draw on the energy-rich bonds of the phosphagen reserve, transferring these to ADP and thus producing extra ATP. Later, after activity has ceased, the metabolic generator continues to run for a short time, so that ATP continues to be produced and, reacting now with the free guanidine base, resynthesizes phosphagen and thus replenishes the store. These notions may be diagrammatically summarized in the so-called 'energy dynamo' of Fig. 25.

The second energy-rich bond of ATP is not directly available as a source of energy for chemical synthesis, nor for the production of mechanical or electrical energy. It can, however, be rendered available through the activity of the peculiar enzyme, myokinase. Unlike enzymes in general, myokinase will tolerate prolonged heating to 100° C. and other kinds of physico-chemical maltreatment. It has been prepared from muscle, and appears to be present in other tissues as well. This enzyme specifically catalyses the transference of the terminal phosphate radical from

one molecule of ADP to a second molecule of the same substance, so that AMP and ATP are produced:



The new molecule of ATP can then be utilized in the usual manner, while the residual molecule of AMP (adenylic acid) becomes liable to attack by a specific adenylic deaminase, which catalyses a hydrolytic deamination of the adenine radical and gives rise to inosinic acid.

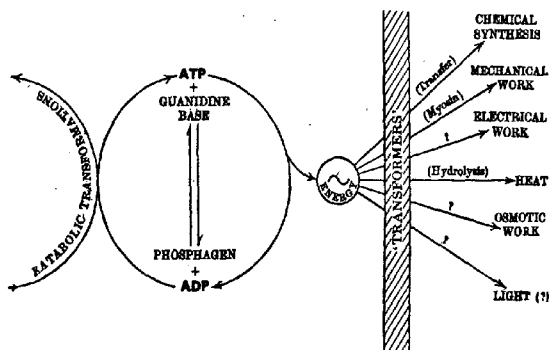
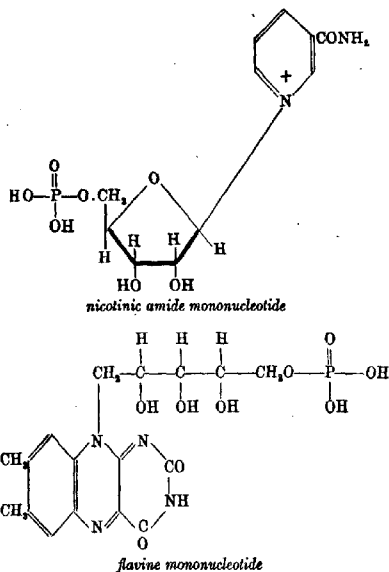


Fig. 25. The energy dynamo (modified after Lipmann).

Adenine compounds also play a central part in metabolic processes that lead to the generation of new energy-rich bonds, quite apart from their function as carriers of these bonds. These reactions, for the most part, are oxidative in nature, and involve the participation of Co I, Co II and adenine-flavine dinucleotide. Adenylic acid enters into the composition of all three of these important compounds, in which it is present in combination with other nucleotides or near-nucleotides. These are, respectively, nicotinic amide mononucleotide and the so-called flavine mononucleotide.

Nicotinic amide mononucleotide is a true nucleotide, the base present being nicotinic amide and the pentose sugar D-ribofuranose.

Flavine mononucleotide is not a true nucleotide, however, although it is usually accorded that title. A nitrogenous base is present in the form of 6-7-dimethyl-iso-alloxazine, but the place of the pentose sugar is taken by the corresponding sugar-alcohol, D-ribitol:



Co I is formed by the combination of a molecule of adenylic acid (adenine mononucleotide) and one of nicotinic amide mononucleotide, the union taking place by condensation between their respective phosphate radicals. In *Co II* a third phosphate radical is present, and this is generally thought to be interposed between the other two. These two compounds are often loosely referred to as the 'diphospho-' and 'triphospho-pyridine' nucleotides respectively.

Adenine-flavine dinucleotide is similarly formed by the condensation of a molecule of adenylic acid with one of the flavine

mononucleotide, and the resulting dinucleotide is known to occur, as, indeed, is flavine mononucleotide itself, as the prosthetic group in certain oxidation catalysts, the behaviour of which we considered in Chapter IV.

We know very little at the present time about the manner in which these important nucleotides and the corresponding nucleosides are synthesized by living cells, but it has recently been discovered by Kalckar that the nucleoside inosine (hypoxanthine-9- β -D-ribofuranoside) is attacked by an enzyme that occurs in rat liver (nucleoside phosphorylase). The mode of attack is not hydrolytic but phosphorylytic, and the products of the reaction, which is freely reversible, are the nitrogenous base, hypoxanthine, and a phosphorylated ribose.

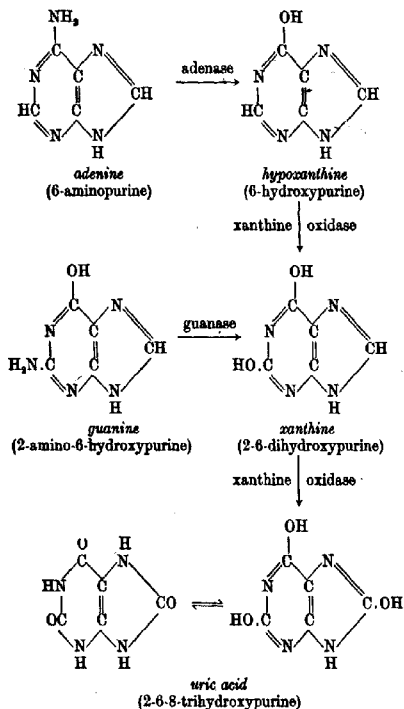
METABOLISM OF PURINE BASES

Adenine (6-aminopurine) occurs in small amounts in the free state and has been isolated from many vertebrate and invertebrate tissues. It also arises by the degradation of nucleic acid. On the synthetic side it undergoes conversion into adenosine, adenylic acid, etc., and is degraded by hydrolytic deamination to yield hypoxanthine under the influence of an enzyme called adenase. Adenase appears to be widely distributed and has been detected in the tissues of numerous animal species.

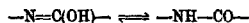
Guanine (2-amino-6-hydroxypurine), like adenine, occurs in nucleic acid, but there is no reason to think that it plays as important a part in cellular metabolism as does adenine. It is an exceedingly insoluble substance and is deposited in crystalline form in special cells, known as iridocytes, in many animals, where it is responsible for the beautiful iridescence of many fishes, for example. Guanine has a special and apparently unique function among spiders, where it replaces uric acid as the predominant product in nitrogenous excretion (p. 273).

Like adenine, guanine is degraded by hydrolytic deamination to yield xanthine. Hypoxanthine and xanthine then undergo serial oxidation under the influence of xanthine oxidase to give

uric acid. These metabolic relationships may be summarized as follows:



In passing, it should be noticed that all the hydroxypurines (often known as 'oxypurines') are tautomeric substances which readily undergo transformation at the



groupings. In the scheme above, only the *enol* forms are given for the sake of clarity, except in the case of uric acid, in which the *keto* form is believed to predominate.

The distribution of adenase, guanase and xanthine oxidase among animal tissues is very erratic. It is said, for example, that man and the rat possess no adenase, though the enzyme is common elsewhere, while the tissues of the embryonic pig are stated to contain guanase, in contradistinction to those of the adult, which do not. Again, xanthine oxidase is present in the liver of most birds, e.g. goose and domestic fowl, but is absent from that of the pigeon (p. 272).

METABOLISM OF URIC ACID

Uric acid may arise from purine bases in animals of any kind, whether they are ammonotelic, ureotelic or uricotelic. Uricotelic animals, as we have seen, convert the bulk of their waste nitrogen into uric acid, but the amounts of uric acid that arise from purine metabolism are relatively very small, accounting perhaps for about 5% of all the nitrogen excreted.

Uric acid is excreted without further chemical manipulation by uricotelic animals, but in most other forms it is more or less extensively degraded before being excreted (see Table 22). The first stage in the process of uricolysis consists in the oxidation of uric acid itself to the more soluble substance allantoin, under the influence of urico-oxidase. This takes place in all non-uricotelic animals apart from man and the higher apes (Primates), while the Dalmatian coach-hound is peculiar among dogs in that it excretes only a small part of its total purines in the form of allantoin. In other mammals, however, allantoin is excreted in place of uric acid, but uricolysis stops at this stage. In most other non-uricotelic animals, allantoin is further degraded to yield allantoic acid, thence to urea, and finally even to ammonia, though in some animal groups the complete set of uricolytic enzymes is lacking. The stages involved in the complete process are summarized in Table 22, together with the names of the enzymes concerned and some indications of their distribution.

It is worthy of note that, like adenase, guanase and xanthine oxidase, the uricolytic enzymes are very erratically distributed among animals. In particular, it is interesting to notice that, with the evolution of more complex forms of life, the tendency,

as far as purine metabolism is concerned, has been to lose old enzymes rather than to acquire new ones, a fact which is amply illustrated by Table 22.

TABLE 22. END-PRODUCTS OF PURINE METABOLISM
(After Florkin and Duchâteau)

End-product	Groups
<chem>O=C1NC(=O)NC(=O)N1</chem> <i>uric acid</i>	Primates Birds Uricotelic reptiles Insects (other than Diptera)
\downarrow urico-oxidase	
<chem>NC(=O)NC(=O)N</chem> <i>allantoin</i>	Mammals (other than Primates) Insects (Diptera only) Gastropods
\downarrow allantoinase	
<chem>NC(=O)NC(=O)C(=O)O</chem> <i>allantoic acid</i>	Fishes (some teleosts)
\downarrow allantoicase	
$\begin{matrix} \text{NH}_2 & \text{COOH} \\ & \\ 2\text{CO} & + \text{CHO} \\ & \\ \text{NH}_2 & \text{glyoxylic} \\ & \text{acid} \end{matrix}$ <i>urea</i>	Fishes (in general) Amphibia Lamellibranchs (fresh water)
\downarrow urease	
$\text{NH}_3 + \text{CO}_2$	Gephyrean worms Lamellibranchs (marine) Crustacea

CHAPTER XIII
ANAEROBIC METABOLISM
OF CARBOHYDRATES: ALCOHOLIC
FERMENTATION

INTRODUCTION

RELATIVELY little has been known about the aerobic breakdown of carbohydrates until recent years, though a great deal was learned about their anaerobic metabolism in yeast and in muscle. It may strike the reader as curious that these two kinds of cells, so different in their organization and function, should have been selected for examination rather than any others. Yeast, however, has long been a matter of great commercial importance for the production of alcoholic beverages and for the manufacture of industrial alcohol. Furthermore, various important by-products of fermentation, such as the components of fusel oil, find many important applications in chemical technology. No wonder, then, that alcoholic fermentation has been extensively studied. In the case of muscle, interest has been aroused by more academic considerations. Muscle does mechanical work. Many muscles can be isolated and made to contract outside the body, and in these, beyond all other tissues, we have the opportunity of measuring the amount of work done by a biological system and attempting to correlate it with the amount of chemical change taking place simultaneously. It was rather late in the history of the subject before it was realized that, in spite of their many apparent differences, yeast and muscle both derive the energy they expend through very similar chemical manipulations of their carbohydrate starting-materials.

We know now that the aerobic metabolism of starch, glycogen and glucose is, so to speak, a continuation of their anaerobic metabolism and, moreover, that it is much more complicated. Here, therefore, we shall deal first with anaerobic and later with aerobic metabolism. Starch, glycogen and glucose provide major sources of energy for plants and animals, to make no mention

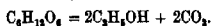
of the innumerable micro-organisms which likewise derive energy and employment from the breakdown of these substances. Their breakdown is attended by the liberation of some at least of the intrinsic energy of the carbohydrate molecule, but how much of the available, or free, energy becomes *biologically* available to any given organism depends upon the nature of the chemical changes the organism is able to accomplish.

When glucose is burned in a bomb calorimeter the heat set free ($-\Delta H$) amounts to about 674,000 cal. per g.mol. The change of entropy ($T \cdot \Delta S$) corresponds to some 12,000 cal., so that the loss of free energy ($-\Delta F$) associated with the complete combustion of 1 g.mol. of glucose is approximately 686,000 cal. The synthesis of 1 g.mol. of glucose, in a green plant or elsewhere, therefore requires the provision of about 686,000 cal. of energy. When an animal oxidizes 1 g.mol. of glucose to carbon dioxide and water, 686,000 cal. of energy become free and available. The biological efficiency of the organism can be measured in terms of the extent to which these 686,000 cal. can be harnessed by the organism and put to use for its biological purposes.

Now glucose can be broken down in other ways than by complete oxidation. Muscle cells, working under anaerobic conditions, can convert glucose into lactic acid, a process known as glycolysis:



Yeast, again, can carry out an anaerobic fermentation of glucose, yielding ethyl alcohol and carbon dioxide:



In neither of these transformations is the change of free energy as large as it is in complete combustion, for a large proportion of the total free energy of the starting material remains in the products, and access to this can only be gained by further degradation of these substances.

The change of free energy associated with complete oxidation of lactic acid amounts to about 325,000 cal. per g.mol., or 650,000 cal. for the 2 g.mol. formed from 1 g.mol. of glucose. Consequently, the loss of free energy associated with glycolysis is less than that associated with complete combustion of glucose

by approximately 650,000 cal. per g.mol. of glucose transformed. The loss of free energy in glycolysis therefore amounts to only $(686,000 - 650,000) = 36,000$ cal. per g.mol. of glucose. Anaerobically, therefore, muscle gains access to only 36,000 cal. as contrasted with the 686,000 cal. which become available when the same amount of glucose is completely oxidized under aerobic conditions. To gain access to the same amount of energy, therefore, a muscle will require to glycolyse nearly 20 times as much glucose as it will if it oxidizes glucose to carbon dioxide and water completely. Aerobic metabolism, in fact, is far more efficient than anaerobic. But in neither case does it necessarily follow that the cell or tissue can actually harness and utilize *all* the energy to which it gains access by oxidizing, glycolysing or fermenting its food materials. How this energy is 'trapped', and how much of it can be 'trapped', we shall see in ensuing chapters, but at the present time we are only at the beginning of a knowledge of biochemical energetics.

It is convenient to classify organisms as aerobic or anaerobic as the case may be. Relatively few living organisms are strictly anaerobic. Indeed, as far as we know, strict anaerobiosis is practically restricted to a few groups of bacteria, and these are not merely unable to utilize oxygen but are actually poisoned by it. The vast majority of micro-organisms are facultative anaerobes, i.e. they can utilize oxygen when it is available and oxidize their foodstuffs completely, but can still survive under anaerobic conditions by catalysing a partial breakdown of the same food materials. Animals for the most part might almost be classified as 'strict' aerobes, since few of them can live for long in complete absence of oxygen. But certain processes can go on in animal tissues under anaerobic conditions, provided the 'oxygen debt' thus incurred can sufficiently soon be repaid.

ALCOHOLIC FERMENTATION

Alcoholic fermentation has been familiar to the human species since prehistoric times, yet it was not until after 1857 that its cause was discovered. In that year Louis Pasteur was studying

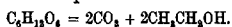
the lactic fermentation of milk and trying to discover its cause. The views held at that time look very strange by modern standards, for the great Liebig, for instance, considered that the nitrogenous constituents of the fermenting mixture reacted with air, setting up 'unstabilizing vibrations' as they did so, and these vibrations were believed to rupture the fermenting molecules. The fact that a new fermentation could be initiated by inoculating the medium with a trace of an already fermenting fluid was attributed to the transference of vibrating material to the new medium.

Pasteur began his experiments with media containing very simple substances such as sugars, ammonium tartrate and mineral phosphates, none of which could reasonably be expected to develop 'unstabilizing vibrations'. His results were simple and clear-cut. Fermentation took place only in the presence of certain microscopic organisms, the lactic acid-producing bacteria of the present day. When precautions were taken to exclude these organisms, no fermentation occurred. Extending his studies to alcoholic fermentation in 1860, Pasteur showed that whenever it took place the appropriate micro-organism, in this case yeast, grew and multiplied. He therefore concluded that fermentation is a physiological process, intimately bound up with the life of the yeast cell. In 1875, having shown that fermentation can take place in complete absence of oxygen, Pasteur defined fermentation as 'Life without oxygen'.

More than 20 years elapsed before the next major step forward, but in 1897 Hans and Eduard Buchner made a key discovery which opened the door not only to the investigation of the mechanisms of fermentation but to the whole of modern enzyme chemistry. Like many other great discoveries, that of the Buchners had in it an element of chance. They were primarily interested in making cell-free extracts of yeast for therapeutic purposes, and this they accomplished by grinding yeast with sand, mixing it with kieselguhr, and squeezing out the juice with a hydraulic press. There then arose the problem of preserving their product. Since it was to be used for experiments on animals the ordinary antiseptics could not be used, so they tried the method usual in kitchen-chemistry of adding large amounts of

sucrose. This led to the momentous discovery that sucrose is rapidly fermented by the yeast juice. Here, for the first time, fermentation was observed in the complete absence of living cells, and at last it was possible to study the processes of alcoholic fermentation independently of all the other processes—growth, multiplication and excretion—which accompany fermentation in the living yeast cell.

The Buchners' work was soon followed by intensive studies of the properties of yeast juice. It was found capable of fermenting glucose, fructose, mannose, sucrose and maltose, all of which are fermented by living yeast. The disaccharides, sucrose and maltose, appear to be hydrolysed to yield their constituent monosaccharides before being fermented. Glucose itself is almost quantitatively converted into ethyl alcohol and carbon dioxide according to the equation



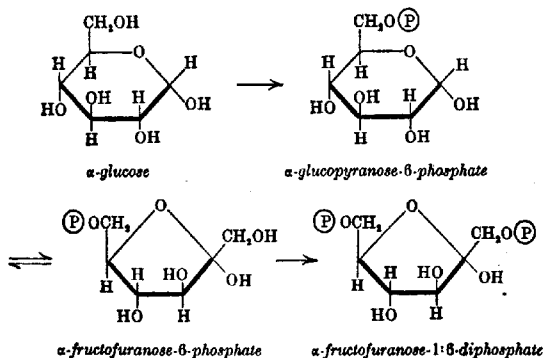
Traces of glycerol are always found among the products.

Fresh yeast juice is much less active than living yeast. The rate of fermentation can be followed by measurements of the rate of evolution of carbon dioxide, and experiments carried out in this way show that living yeast works 10–20 times as fast as an equivalent quantity of yeast juice. Moreover, the fermentative power of the yeast juice falls off rapidly with time. The juice is not inactivated by drying at 30–35° C. or by the addition of chloroform, but loses its activity if heated to 50° C., suggesting that enzymes must be involved.

The first important step towards analysing the mode of action of yeast juice was made by Harden and Young in 1905. If fresh yeast juice is added to a solution of glucose at pH 5–6, fermentation begins almost at once. The rate of carbon dioxide production presently falls off, but can be restored by the addition of inorganic phosphate. The recovery is only temporary, however; the added phosphate disappears, and the rate of fermentation falls off as the concentration of free phosphate declines. The addition of more phosphate produces another burst of fermentation and so on.

The disappearance of added inorganic phosphate from fermenting mixtures suggested that organic phosphate esters must

probably be formed and, as Harden and Young showed, this is indeed the case, for they were able to isolate such an ester in the form of fructofuranose-1:6-diphosphate ('hexose diphosphate'). This substance, like glucose, is fermented if added to an actively fermenting system, and must probably be an intermediate in the process of fermentation. Later Robison isolated another sugar phosphate, this time a monophosphate which, on detailed examination, proved to consist of an equilibrium mixture of glucopyranose-6-phosphate and fructofuranose-6-phosphate. Like hexose diphosphate these esters are fermentable. It seemed clear that these substances must arise by the coupling of inorganic phosphate with glucose, the respective esters probably arising in the following order:



How these esters are formed, and in what way the fructose diphosphate is eventually converted into alcohol and carbon dioxide, are questions which were only answered over a period of decades and by the efforts of many workers in many different countries. Certain stages were first elucidated by studies of muscle extracts, for it became clear in time that the fermentation of glucose by yeast juice runs closely parallel to the glycolysis of glycogen by suitable muscle extracts. Among the names that stand out in connexion with the further analysis of fermentation and glycolysis are those of Embden, Neuberg, Meyerhof, Parnas,

D. M. Needham and Cori, but these are only a few of the many who have contributed.

The next fundamental step forward was also made by Harden and Young when they discovered that yeast juice loses its activity if dialysed. Activity could be restored to dialysed juice, either by adding the dialysate, or by means of small quantities of boiled juice. This showed that, in addition to enzymes, yeast juice contains dialysable, thermostable substances which function as co-enzymes. Yeast juice thus came to be regarded as consisting of 'zymase', a non-dialysable, thermolabile enzyme, plus 'cozymase', a dialysable, thermostable fraction. We know now, of course, that zymase is in reality a complex mixture of enzymes and that cozymase consists not of one substance only but of several.

It is neither possible nor desirable here to give an historical account of subsequent work on the problem of fermentation. There were mistakes and gaps in the schemes that replaced one another in quick succession during the ensuing years, but one by one the mistakes were rectified and the gaps filled in until, at the present time, we have what we believe to be a clear picture of most of the details of the process. Before studying this picture it is necessary to know something more about the composition of cozymase.

Cozymase comprises a number of factors. *Co-carboxylase*, now known to be identical with the pyrophosphate of vitamin B₁ (aneurin, thiamine), is the coenzyme of carboxylase, an enzyme which catalyses the 'straight' decarboxylation of pyruvic acid to form acetaldehyde and carbon dioxide. *Co 1* (also known as cozymase, or sometimes as cozymase 1) is identical with the adenine-nicotinic amide dinucleotide which we have discussed already. In fermentation, as in respiration, this substance functions in collaboration with certain dehydrogenases as a hydrogen acceptor, donator and carrier. *Adenosine triphosphate*, which we have discussed, acts as a phosphate carrier: as will be remembered, the terminal phosphate radical is bound to the rest of the molecule by an energy-rich bond, the energy associated with which can be transferred, along with the phosphate radical itself, to other substances. *Magnesium ions*, too, are involved. They function

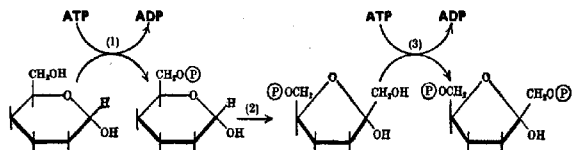
as activators for many enzymes concerned with phosphate metabolism and, in particular, for enolase, an adding enzyme. In addition to these 'classical' coenzymes, dialysis removes other substances, including the ions of *inorganic phosphate*, *calcium* and *potassium*, and evidence is accumulating to show that, for certain reactions at least, even these substances are of great importance and may strictly be classified as coenzymes of fermentation.

Since all of the co-substances are essential components of the fermenting systems of yeast juice, it follows that the breakdown brought about by dialysis is due, not to the abolition of some one particular reaction, but of many. Dialysed juices, with and without the addition of one or more of the known coenzymes, have therefore played a large part in unravelling the intricate reaction sequence that underlies fermentation. Much further information has been gained by taking advantage of the fact that certain substances have empirically been found to slow down or stop particular reactions. The addition of these inhibitors leads to the accumulation of intermediate products which can be isolated and identified. The reagents most widely used for this purpose have been *sodium bisulphite*, *sodium fluoride* and *sodium iodoacetate*.

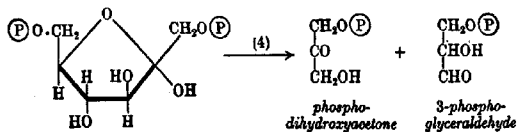
For purposes of discussion the reaction sequence of fermentation can be arbitrarily divided into several stages, each of which involves one or more individual chemical operations, but because we can dissect the whole process into stages and steps in this way, it is not to be supposed that fermentation as such is a step-by-step process, catalysed by a mere mixture of enzymes. Fermentation is a highly organized procession of chemical events, the overall result of which is the decomposition of glucose, with production of alcohol and carbon dioxide, together with the provision of energy which enables the cells to carry out the synthesis of the new tissue materials required for their maintenance, growth and reproduction.

(i) *Formation of Phosphorylated Sugars.* We have already seen that several sugar phosphates can be isolated from fermenting systems to which glucose and inorganic phosphate have been added. If glucose and inorganic phosphate are added to a

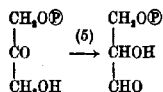
dialysed juice, however, there is no fermentation and no sugar esters are formed, showing that one or another of the coenzymes must play a part in their synthesis. If ATP is added to the dialysed juice, however, phosphorylation of the sugar begins again, and fructofuranose-1:6-diphosphate, fructofuranose-6-phosphate and glucopyranose-6-phosphate can be isolated from the system. Work with highly purified enzymes has resolved this stage into three separate reactions. First of all one phosphate radical is transferred from ATP to glucose, yielding glucopyranose-6-phosphate and ADP, a process which is catalysed by *hexokinase* (reaction 1). Next the glucose ester is reversibly converted into fructofuranose-6-phosphate (reaction 2), the catalyst being *oxoisomerase*. A phosphate radical is then transferred from a second molecule of ATP to the fructose mono-ester, yielding fructofuranose-1:6-diphosphate. This reaction (reaction 3) is catalysed by *phosphohexokinase*. This group of reactions may be summarized as follows, writing only the forward reactions for the sake of clarity:



(ii) *Splitting of the Hexose Chain*. If glucose or one of the intermediate esters is added to yeast juice in the presence of iodoacetate, small amounts of 'triose phosphate' can be isolated, showing that hexose diphosphate is split into two 3-carbon fragments which, on isolation, prove to consist of an equilibrium mixture of 3-phosphoglyceraldehyde and phosphodihydroxyacetone. The enzyme concerned, *zymohexase*, has been isolated, and the reaction it catalyses (4) shown to be reversible:



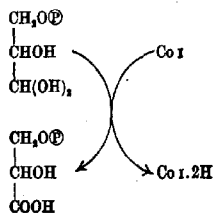
Of the two components of triose phosphate the 3-phosphoglyceraldehyde is the more important from our point of view, since its derivatives appear lower down in the reaction chain, whereas no direct derivatives of phosphodihydroxyacetone are found. But dihydroxyacetone phosphate is not lost to the system, which contains a powerful *phosphotriose isomerase*. This enzyme catalyses the interconversion of the two triose phosphates (reaction 5):



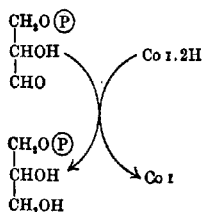
The original hexose has now been phosphorylated and quantitatively split into phosphorylated triose.

(iii) *Oxidation of Phosphoglyceraldehyde*. If hexose diphosphate or 'triose phosphate' is added to yeast juice in the presence of fluoride, two further phosphorylated derivatives of glycerol accumulate, viz. phosphoglycerol and phosphoglyceric acid in equimolecular proportions. On isolation and examination the acid proves to be an equilibrium mixture of 3- and 2-phosphoglyceric acids. Of these the primary product must presumably be the 3-compound since it is formed from 3-phosphoglyceraldehyde. These products arise by the oxidation of one molecule of phosphoglyceraldehyde at the expense of the reduction of another.

Yeast juice contains a powerful *triosephosphate dehydrogenase*, an enzyme which requires Co I, which is also present. But the amount of coenzyme is very small, and the whole would soon become reduced by acting as hydrogen acceptor for the oxidation of phosphoglyceraldehyde (reacting in its hydrated form):



Once all the available coenzyme had been reduced in this way, fermentation would come to an end. But yeast contains also an *α-glycerophosphate dehydrogenase*. This dehydrogenase also co-operates with Co I and, like dehydrogenases generally, can act reversibly. The reduced coenzyme can therefore become re-oxidized by passing on its 2H to a second molecule of phosphoglyceraldehyde, which is thereby reduced to phosphoglycerol:



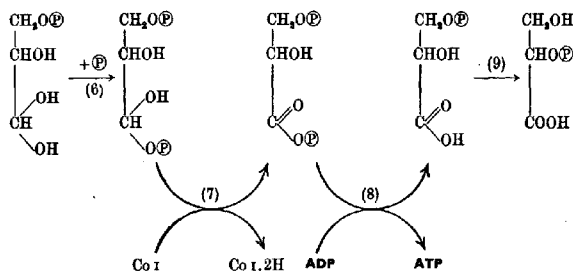
As a result of this operation the reduced coenzyme becomes reoxidized and available, therefore, for the oxidation of another batch of phosphoglyceraldehyde.

It may be pointed out in passing that the reduction of the aldehyde to phosphoglycerol is not normally a large-scale process, but one that only takes place under unusual circumstances such, for instance, as when the system is poisoned with fluoride, and the reasons for this we shall discover presently.

In recent years it has been found that the oxidation of phosphoglyceraldehyde is a considerably more complex process than was formerly supposed. It was observed by Meyerhof and by D. M. Needham, independently and at the same time, that when phosphoglyceraldehyde is oxidized to phosphoglyceric acid in muscle extracts, one molecule of ATP is synthesized for every molecule of phosphoglyceric acid formed. A similar phenomenon could also be observed in yeast-juice fermentation, and attempts were made to discover its cause, using highly purified (crystalline) triosephosphate dehydrogenase. It was then found that no oxidation of phosphoglyceraldehyde took place except in the presence of inorganic phosphate. When the latter was added, however, a brisk oxidation took place, one molecule of phosphate

disappearing for every molecule of phosphoglyceraldehyde oxidized. The product of oxidation now proved to be, not 3-phosphoglyceric acid, but a new compound, 1:3-diphosphoglyceric acid.

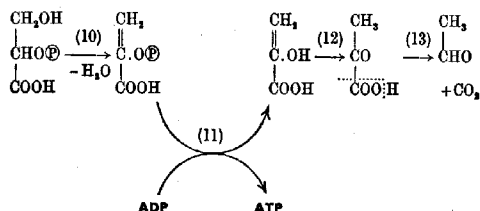
To account for the disappearance of inorganic phosphate in a yeast-juice fermentation it may therefore be supposed that an intermediate 1:3-diphosphoglyceraldehyde is first formed (reaction 6) and then undergoes dehydrogenation (7) under the influence of *triosephosphate dehydrogenase*, yielding 1:3-diphosphoglyceric acid. Reaction 7 is inhibited by iodoacetic acid, which blocks the —SH groups of the dehydrogenase. The next stage consists in the transference of the phosphate radical at position 1 to a molecule of ADP, so that 3-phosphoglyceric acid and ATP are formed (reaction 8), the process being catalysed by an unnamed *phosphokinase*. The 3-phosphate is then converted into the 2-ester (reaction 9) through the agency of *phosphoglyceromutase*. The reactions, all of which are reversible, may be written in the following manner:



(iv) *Dephosphorylation of Glyceric Acid.* If one or both of the phosphoglyceric acids is added to whole yeast juice it undergoes fermentation. If, however, a dialysed juice is used there is no fermentation, but a new intermediate accumulates, viz. phosphoenol-pyruvic acid. This arises by the dehydration of 2-phosphoglyceric acid (reaction 10) at the hands of *enolase*. It is at this point that fluoride inhibits fermentation; it does so because *enolase* requires magnesium ions for activity and is, apparently, a magnesium protein. Fluoride forms a complex magnesium

fluorophosphate in the presence of inorganic phosphate. Dialysis, as ordinarily performed, does not stop enolase activity; it does, however, remove the coenzyme required for the next reaction, viz. the decomposition of phosphopyruvic acid.

If ADP is added to a dialysed juice containing phosphopyruvic acid, the latter begins to break down, and pyruvic acid appears. This reaction (11) is catalysed by an unnamed *phosphokinase*, and the phosphate radical is transferred to ADP, yielding ATP once again:



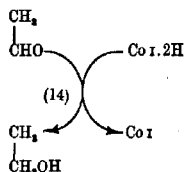
The *enol*-pyruvic acid liberated in reaction 11 may pass over into the more stable *keto*-form (reaction 12); it may be, however, that the less stable and therefore more reactive *enol*-form enters the next reaction as fast as it is produced.

Pyruvic acid accumulates in a dialysed extract provided with ADP and phosphopyruvic acid. It does so because the next reaction, in which the pyruvic acid is decarboxylated, requires the presence of *co-carboxylase*, together with the enzyme *carboxylase*. The products of this reaction (13) are carbon dioxide and acetaldehyde, and the formation of the latter can be demonstrated by adding sodium bisulphite to a fermenting mixture, when the addition compound, acetaldehyde-sodium bisulphite, $\text{CH}_3\text{CH}(\text{OH})\text{SO}_3\text{Na}$, is formed and can be isolated. The splitting of pyruvic acid is probably the only irreversible process in the whole fermentation sequence, apart from the initial phosphorylations (reactions 1, 3).

(v) *Production of Alcohol*. The final stage of the process consists in the reduction of acetaldehyde to ethyl alcohol, and the mechanism of the reaction requires special consideration.

It will be remembered that in the presence of fluoride, phosphoglyceric acid and phosphoglycerol are produced. Phosphoglyceric acid is formed from phosphoglyceraldehyde by reactions which involve the reduction of Co I. The reduced coenzyme passes on its 2H to another molecule of phosphoglyceraldehyde since, without this, the whole of the available coenzyme would soon become and remain reduced. As no more phosphoglyceric acid could then be formed, fermentation would speedily come to an end.

In a normal, as opposed to a fluoride, fermentation, a second molecule of phosphoglyceraldehyde is not required at this point, for there is available an alternative hydrogen acceptor in the form of acetaldehyde. Under the influence of *alcohol dehydrogenase*, working 'in reverse', the 2H of the reduced coenzyme are transferred instead to acetaldehyde, alcohol is formed, and the oxidized form of Co I is regenerated and can be used over again (reaction 14). This final operation can be written as follows:



Reaction 14, like 7, is inhibited by iodoacetate, which blocks the —SH groups of alcohol dehydrogenase.

The overall results of this reaction sequence, which is summarized in Fig. 26, are, first, that for each molecule of glucose fermented, two molecules of alcohol and two of carbon-dioxide are formed. Secondly, for each molecule of phosphoglyceraldehyde oxidized, one molecule of Co I is reduced, and later reoxidized at the expense of the molecule of acetaldehyde formed from the phosphoglyceraldehyde, so that the coenzyme finishes in the oxidized condition in which it began. Thirdly, two molecules of ATP are dephosphorylated in the phosphorylation of each molecule of glucose. Each molecule of the phosphorylated product,

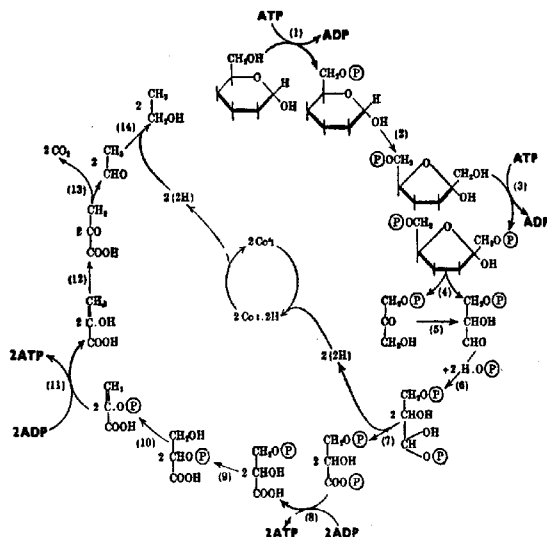


Fig. 26. Scheme to summarize reactions of alcoholic fermentation of glucose by yeast juice. The reactions are numbered to correspond to the description given in the text. For names of enzymes, coenzymes and inhibitors, see Table 23.

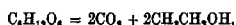
TABLE 23. ALCOHOLIC FERMENTATION: ENZYMES, COENZYMES AND INHIBITORS
(see also Fig. 26)

Reaction	Enzyme	Coenzyme	Inhibited by
1	Hexokinase	ATP	Dialysis
2	Oxoisomerase		
3	Phosphohexokinase	ATP	Dialysis
4	Zymohexase		
5	Phosphotrioseisomerase		
6	? Spontaneous	$-\text{PO}_3\text{H}_2$	Dialysis
7	Triosephosphate dehydrogenase	Co I	Dialysis; $\text{CH}_3\text{I}, \text{COOH}$
8	Unnamed phosphokinase	ADP	Dialysis
9	Phosphoglyceromutase		
10	Enolase	Mg ions	NaF
11	Unnamed phosphokinase	ADP	Dialysis
12	? Spontaneous		
13	Carboxylase	Coccarboxylase	Dialysis
14	Alcohol dehydrogenase	Co I	Dialysis; NaHSO_4 ; $\text{CH}_3\text{I}, \text{COOH}$

fructose-1:6-diphosphate, yields two molecules of 3-phosphoglyceraldehyde, and each of these takes up a molecule of inorganic phosphate before being oxidized. After oxidation has taken place, the phosphate radicals, two for each molecule of glucose entering the system, are returned in the form of ATP in reaction 8, so that, at this stage in fermentation, the yeast has just recovered the amount of ATP used in the first stages. Presently, however, two more molecules of ADP are taken in and, from these, two fresh molecules of ATP are formed in reaction 11. Thus, as far as the ADP/ATP system is concerned, *two new molecules of ATP are gained for each molecule of glucose fermented.*

ENERGETICS OF FERMENTATION

Now let us recall the overall equation of alcoholic fermentation:



The loss of free energy in this reaction is roughly 50,000 cal. per g.mol. glucose fermented. Two new energy-rich bonds are formed at the cost of one molecule of glucose. Now each of these energy-rich bonds represents some 10,000 cal. of *immediately available free energy*. It follows that of the 50,000 cal. or thereabouts which become available when a gram-molecule of glucose is fermented, 20,000 cal. are transferred from their source, glucose, to the energy-rich bonds of adenosine triphosphate, *the only known source of energy that can be directly utilized by living organisms*. About 40 % of the total free energy lost when glucose undergoes fermentation is thus rendered immediately accessible to the cell. That part of the energy which is not transferred from glucose to ATP is degraded in the form of heat, in part at any rate, and this, probably, is why the temperature of a fermenting liquor is always rather higher than that of its surroundings. This, however, is not altogether disadvantageous, since, within limits, fermentation, growth and multiplication all proceed more rapidly at higher temperatures.

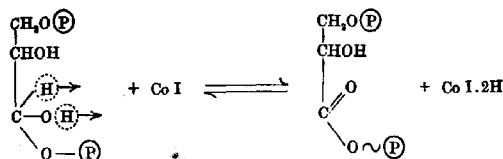
The question is often asked, why is it that yeast does not break up glucose into alcohol and carbon dioxide directly, instead of in this rather complicated manner? The *total free-energy*

yield of the process would be the same, no matter how the sugar was fermented, but, by working in the way it does, the yeast is able to transfer a large proportion of the total free energy of the process to the directly utilizable energy-rich bonds of ATP. If the glucose were *directly* split, even if enzymes existed that could catalyse this process, the chances are that the vast bulk of the free energy that fermentation renders available would be degraded as heat, and thus lost to the system.

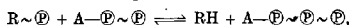
Let us now see how this important transference of chemical energy from one place to another is achieved. Living cells seem never to have discovered enzymes capable of catalysing a complete split of the 6-carbon chain of unmodified glucose. The preliminary phosphorylation reactions seem, therefore, to be devices for getting glucose into a metabolizable form. To accomplish this, chemical work has to be done, and is carried out at the expense of the terminal energy-rich phosphate bonds of two molecules of ATP (reactions 1, 3). Then, and only then apparently, the 6-carbon chain can be ruptured. In the subsequent metabolism of the products, the energy originally put in is recovered (reaction 8) and the energetic *status quo* is re-established. Later, still more energy becomes available (reaction 11), and may be used to start off the fermentation of fresh molecules of glucose, for example, or for the synthesis of new and complex tissue materials, so that the cells may grow and, in due time, divide.

The resynthesis of ATP from ADP requires the provision of some 10,000 cal. of free energy per gram-molecule. This is provided by the generation of new energy-rich bonds in the partial-breakdown products of glucose. The first new bond appears when the presumptive 1:3-diphosphoglyceraldehyde is oxidized to the corresponding acid (reaction 7). This, it will be remembered, is a process of dehydrogenation, and the removal of 2H from the aldehydic molecule results in structural changes within the molecule. These are attended by a redistribution of the intrinsic energy of the system, leading to a concentration of energy in the bond which links the phosphate radical in position 1; in other words, to the generation of a new energy-

rich bond. The process can be represented in the following manner:

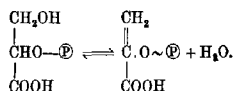


The free energy of the new bond has been estimated at about 11,250 cal., i.e. rather more than is required to forge a new ATP bond, so that in the subsequent transfer reaction,



the reaction swings far over towards the right-hand side.

Further energy-rich bonds are generated later when 2-phosphoglyceric acid loses water to yield phospho-*enol*-pyruvic acid (reaction 10):



Here, as before, a chemical change, this time a dehydration, leads to redistribution of the internal energy of the molecule; another new energy-rich bond is generated, and this, like the former, is transferred almost intact to a molecule of ADP, yielding ATP once again.

FERMENTATION BY LIVING YEAST

Yeast-juice fermentation differs from fermentation by live yeast cells in several noteworthy respects. In the first place, the juice is far less active than intact cells. This is probably because the enzymes and coenzymes are not arranged at random in the cell, as presumably they must be in the extract, but in some definite, orderly manner. It is probably safe to assume that, in the yeast cell, as in a factory, the machinery is arranged in a manner calculated to yield the greatest possible degree of efficiency, and it may not be going too far to suggest that the organization is such that the substance produced by one enzyme in the series is

passed immediately on to the next. We know virtually nothing about the internal organization of this or of any other kind of cell, but that an organization of a high order of complexity exists can hardly be doubted.

A second important difference between yeast and yeast juice lies in the effect of inorganic phosphate upon fermentation in the two cases. As Harden and Young first showed, yeast juice can only ferment sugar so long as there is free inorganic phosphate in the medium. The reason for this is clear from what we now know about the mechanisms involved, for inorganic phosphate is required for the conversion of phosphoglyceraldehyde into the diphosphoglyceraldehyde which is the true substrate of the so-called triosephosphate dehydrogenase. The important oxidation process (reaction 7) therefore ceases when inorganic phosphate is not available. Any free phosphate that is present is taken up (reaction 6) and transferred by way of the diphosphates of glyceraldehyde and the corresponding acid to ADP (reactions 7, 8), and the ATP so formed is used to esterify more glucose (reactions 1, 3). If inorganic phosphate is added to a juice fermentation, therefore, it disappears and is replaced by the organically bound phosphate of the sugar esters. But the addition of inorganic phosphate has no effect on the rate of fermentation of sugar by intact yeast cells. Once again the notion of intracellular organization has to be invoked: one must suppose that the interior of the cell is so arranged that inorganic phosphate is always available in the cell at the right place and at the right time.

In the intact cell, we must believe, ATP synthesized by the cell's fermentative activities is utilized for the performance of work of various kinds, so that the terminal phosphate units of ATP are set free again in one way or another. This inorganic phosphate is caught up by the fermentation machine, recharged, so to speak, and again returned to ATP, and so on; a continual cycle of phosphate is built up and used to transfer energy obtained by fermentation to the places at which it is required and, presently, is actually put to employment.

Bound up with the phosphate effect there is an interesting phenomenon known as the arsenate effect. If arsenate is added

to a juice fermentation that has stopped through lack of phosphate, a long-continuing but very slow fermentation begins. This is because arsenate is able to replace phosphate in reaction 6, so that an arseno-phosphoglyceraldehyde is formed and is then oxidized in reaction 7. The product fails, however, to react with ADP in reaction 8, but 1-arseno-3-phosphoglyceric acid is rather unstable and breaks down slowly, liberating arsenate, so that 3-phosphoglycerate is slowly produced. This re-enters the reaction sequence at reaction 9 so that a slow fermentation takes place. An important feature of this arsenate effect is that reaction 8 is by-passed, so that the energy-rich bonds normally generated at this stage are no longer available to the system.

The final products of fermentation, by yeast cells or by juice, always include small quantities of glycerol and other substances. The formation of glycerol can be accounted for in terms of the reactions known to take place in fermentation. At the very beginning of fermentation, glucose is phosphorylated and split to yield phosphoglyceraldehyde. If fermentation is to proceed, this phosphoglyceraldehyde must be oxidized to form phosphoglyceric acid, a process in which Co I is reduced. As yet, no acetaldehyde has been formed by the reduction of which reduced Co I can be reoxidized and so put back into commission. But, as we have learned from experiments on fluoride inhibition, phosphoglyceraldehyde can be used instead of acetaldehyde, and this does in fact take place until some acetaldehyde has been produced. Even when acetaldehyde is being formed, however, small amounts of phosphoglyceraldehyde continue to be reduced, for the system contains an α -glycerophosphate dehydrogenase. The acetaldehyde/alcohol dehydrogenase system gets the lion's share of the reduced coenzyme, partly because acetaldehyde is more readily reduced than is phosphoglyceraldehyde, and partly because the alcohol dehydrogenase is more abundant than the glycerophosphate enzyme. A small proportion of the reduced coenzyme nevertheless reacts with phosphoglyceraldehyde so that a little phosphoglycerol is formed. This is then hydrolysed by a phosphatase that occurs in the yeast, and glycerol itself is set free.

FERMENTATIVE MANUFACTURE OF GLYCEROL

Glycerol is a very important article of commerce, especially in time of war when large amounts are used in the manufacture of explosives. In ordinary times, the glycerol of commerce is a by-product from the manufacture of soaps by the saponification of fats, and fats are always in short supply in war-time. During the war of 1914-18 the British blockade led to a serious fat shortage in Germany, and the resultant shortage of glycerol meant a shortage also of explosives. The problem was met by making use of the ability of yeast to form glycerol.

High yields of glycerol can be obtained from sugar by modifying the course of normal fermentation in either of two ways. The two modified forms are known as Neuberg's 'second' and 'third' forms of fermentation respectively, the 'first' form being normal alcoholic fermentation. In Neuberg's second form, sodium bisulphite is introduced into the fermenting liquors. This gives an addition-compound with acetaldehyde, thus depriving the cells of their normal hydrogen acceptor for the reoxidation of reduced Co I. Its place is taken by phosphoglyceraldehyde, and one molecule of phosphoglycerol is accordingly formed for each molecule of phosphoglyceric acid. The phosphoglycerol is hydrolysed by the yeast phosphatase, while the phosphoglyceric acid continues along the usual path until acetaldehyde is formed, and reacts with bisulphite. Each molecule of glucose therefore yields one molecule of glycerol and one each of carbon dioxide and the aldehyde-bisulphite addition compound. The process is sketched out in Fig. 27*b*, which may be compared with Fig. 27*a*, which represents normal fermentation in similar terms.

The third form of fermentation sets in if the fermenting liquors are made and kept alkaline. Under alkaline conditions, acetaldehyde is no longer reduced to alcohol in the normal manner, but instead undergoes a dismutation. One molecule is oxidized to acetic acid and a second simultaneously reduced to alcohol, and this takes place quite independently of the normal reactions. Acetaldehyde, in fact, is no longer available for the reoxidation of reduced Co I, and its place in that reaction is again taken by

phosphoglyceraldehyde. In this case, therefore, each molecule of glucose gives rise to one of glycerol and one of acetaldehyde, one-half of which is further transformed into acetic acid and the other into ethyl alcohol (Fig. 27c).

In the third form of fermentation, which takes place only in alkaline solutions, the yeast cell changes its metabolism in such

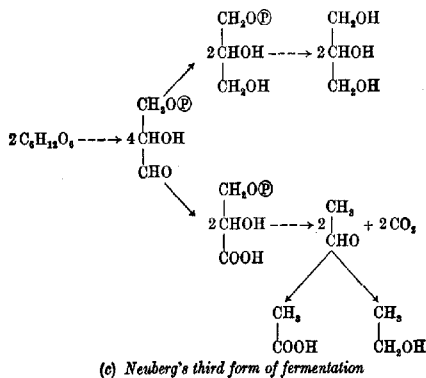
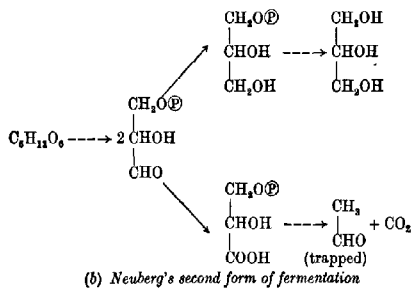
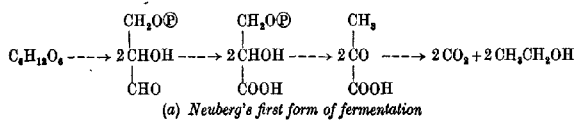


Fig. 27. Neuberg's three 'forms' of fermentation.

a manner as to produce an acid. Unless steps are taken to maintain the alkalinity of the medium, therefore, the pH falls until the medium becomes faintly acid, when the normal form of fermentation reasserts itself and no more acid is produced. We are accustomed to the idea that the environments of living organisms can bring about changes in those organisms, and in the present case the effect of alkalinity in the medium is to change the metabolism of the organism. But the organism reacts by producing acid, and we have therefore a case in which the organism also produces changes in its environment. And this is not by any means the only example of its kind: many bacteria tend to produce acids when cultivated in alkaline media and strongly basic amines when the media are acid (p. 152), so that, in either case, the pH of the medium is changed towards physiological neutrality.

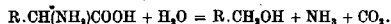
PRODUCTION OF FUSEL OIL

Alcoholic fermentation carried out by live yeast is attended by the production of a number of alcoholic substances other than ethyl alcohol and glycerol, and to these the collective name of 'fusel oil' is applied. These substances usually account for less than 1% of the total alcohols, but are of considerable industrial importance. They are interesting, too, because they are largely responsible for the characteristic flavours and bouquets of alcoholic beverages. Heavy wines, such as port, contain considerable amounts of higher alcohols, especially *iso*-amyl alcohol, and these are responsible not only for the taste of the wine but also, in large measure, for the unpleasant effects of over-indulgence, since the higher alcohols are powerful narcotics. Another interesting product of the same kind is the bitter principle of beer: this again is an alcohol, in this case tyrosol.

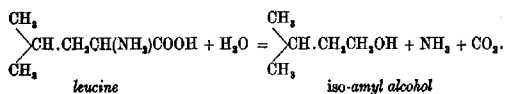
These alcohols arise from amino-acids. The crude liquor contains amino-acids arising from grapes, hops and the like, and more are contributed by the autolysis of dead yeast cells. They are deaminated, apparently to furnish ammonia for the synthesis of new yeast proteins required as the cells grow and multiply, for

if ammonium salts are added to the liquor there is a marked fall in the yields of fusel oil.

Yeast deaminates amino-acids in a peculiar manner that is perhaps unique, the process consisting in an apparently simultaneous decarboxylation and hydrolytic deamination:



In this way the leucines, for example, give rise to the corresponding amyl alcohols, while valine yields *iso*-butyl alcohol, e.g.



Tyrosol arises in the same way from tyrosine.

CHAPTER XIV

ANAEROBIC METABOLISM OF CARBOHYDRATES: MUSCLE AND LIVER

INTRODUCTION

MODERN muscle biochemistry was founded at the beginning of the present century. Before that time, muscular contraction or any other kind of cellular activity was thought to depend upon the sudden decomposition of the large molecules of a hypothetical stuff called 'inogen'. In the case of muscle, this 'inogen' was supposed to give rise to carbon dioxide and L-(+)-lactic acid, furnishing the energy expended by the muscle. It was already well known that muscles produce carbon dioxide when they contract, and that lactic acid is produced in greater or smaller amounts at the same time.

'The justification for considering muscle tissue especially, out of all the active tissues of the organism, lies in the fact that only in muscle can we come near to comparing the chemical changes going on with the simultaneous work done or the energy set free as heat. It is difficult to assess the work performed by a secreting gland, and the metabolism of such an organ can only be studied in elaborate perfusion experiments; great advances have been made in the study of nerve tissue, but here the changes going on are so small as to make their detection only lately possible by modern methods. But certain muscles, and a variety of them, can be removed from the body with absolutely no injury, and can be kept functional for days' (D. M. Needham).

Many different methods have been used to elucidate the problems of muscular contraction. Histology, physiology, biochemistry and X-radiography have all played a part. From the point of view of the histologist we can distinguish between three main types of muscle. These are: (1) the striped or striated voluntary muscle of the skeletal system, (2) the plain, unstriated or involuntary muscle of the visceral system, and (3) cardiac muscle. Most of the chemical work has been done on skeletal

muscle, but there seem to be few differences between the different types from the point of view of the chemistry of the contractile processes.

The structural unit of striated muscle is the *myofibril*, a spindle-shaped little cell. Several of these go to make up a muscle fibre, and many fibres to make up a whole muscle. Under the microscope the muscle fibre shows alternating light and dark bands, or transverse striations. The differences between the appearances of the two kinds of bands are due to differences in their optical properties. The dark-looking striations or *anisotropic bands* show strong double refraction, and when the muscle contracts it can be seen that only these anisotropic regions undergo shortening. The light-looking *isotropic bands*, which are not doubly refracting, do not.

Chemically speaking, muscle consists chiefly of the muscle protein *myosin*, apparently in the form of a gel which accounts for 75-80% of the whole muscle substance. Now myosin is a protein of which the molecule is a rod-shaped or 'fibrous' particle. A solution of myosin is singly refracting when at rest, when the molecules have a purely random distribution. But if the solution is made to flow along a glass tube, the rod-shaped particles all become orientated in the same direction, pointing in the direction of flow, and the solution becomes doubly refracting. It is therefore probable that the anisotropic regions of the muscle fibre are doubly refracting because all the myosin molecules point in the same direction, while in the singly refracting, isotropic regions they lie perhaps entirely at random. There is now abundant evidence to show that the molecule of myosin, like that of keratin, possesses contractile properties, and we know too that only the anisotropic regions shorten when a muscle contracts. It therefore seems likely that the contraction of the muscle as a whole is in reality due to summation of the individual molecular contractions of all the myosin particles which, lying parallel to one another, account for the double refraction of the anisotropic, contractile bands.

This is a rough picture of what is generally regarded as the contractile machinery. We now have two main problems to

consider. First, what is the chemical source of the energy which is expended when the muscle machine does its work and, secondly, how is this energy transformed into the mechanical energy of contraction? At the present time we have a considerable amount of information on the first of these points, but only a few intriguing hints about the second.

The first really significant experiments on the chemistry of muscular contraction were carried out by Fletcher and Hopkins and published in 1907. Working on frog muscles, they showed that larger or smaller amounts of lactic acid are formed when muscle contracts. The general plan of the experiments was as follows. The sartorius or gastrocnemius muscles were removed from the hind legs of a frog and kept under identical conditions. One of the pair was made to do work by being stimulated, and this, the experimental muscle, and the unstimulated control were then dropped into ice-cold alcohol and finely ground with sand. These workers realized that a muscle is capable of doing a very large amount of work in a very short period of time, and that to injure a muscle amounts to stimulating it. By using small muscles and extracting with ice-cold alcohol it was possible to inactivate the muscle enzymes very rapidly indeed, and so to prevent the large-scale chemical changes which would otherwise result from injuries inflicted in the process of grinding. The chemical changes corresponding to the work done by the experimental muscle could then be found by analysing and comparing the extracts with those of control muscles. Other workers had done similar experiments already, but no precautions were then taken to cool the muscle before grinding, with the result that little difference could as a rule be detected between the experimental and control muscles, so grievous is the injury inflicted by grinding.

Fletcher and Hopkins, with their new technique, confirmed and extended the older observation that lactic acid is formed when muscle contracts, and their results demonstrated with beautiful clarity the following points. (i) Muscle can contract in a perfectly normal manner in complete absence of oxygen. (ii) Lactic acid is produced during anaerobic contraction, and

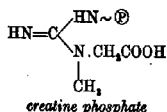
piles up with continued stimulation until, in the end, the muscle becomes fatigued. (iii) If the fatigued muscle is then put into oxygen it recovers its ability to contract, and lactic acid simultaneously disappears. (iv) Less lactic acid is formed in a muscle that is allowed access to oxygen than in one which works anaerobically.

Shortly afterwards it was shown by Meyerhof that the lactic acid is formed from glycogen and that, under anaerobic conditions, the amount of lactic acid formed is chemically equivalent to the quantity of glycogen broken down. A mass of later work made it clear that there is strict proportionality between the amount of work done, the heat produced, the tension developed in a muscle, and the quantity of lactic acid formed, and by 1927 it had become evident that the energy expended in muscular contraction comes from the conversion of glycogen to lactic acid.

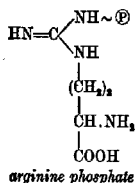
A good deal of interest centred round the phosphate compounds present in muscle, for it was already clear that phosphates play an important part in muscle glycolysis, just as they do in fermentation. The method in general use for extracting phosphates from muscle tissue consisted in chilling the material thoroughly and extracting it with ice-cold trichloroacetic acid or some other protein precipitant. Ice-cold conditions were used here, as in the original work on lactic acid formation, in order to inactivate the muscle enzymes as rapidly as possible. Once the extract had been prepared it was allowed to warm up, and estimations of the phosphate content were subsequently made.

In 1927, however, it was discovered that the ice-cold filtrates from trichloroacetic precipitation contain a hitherto undetected phosphate compound. This substance is exceedingly rapidly hydrolysed in acid solution and had not previously been noticed for that reason. In order to detect and estimate it, the trichloroacetic filtrate must be kept ice-cold until it has been neutralized to a pH of about 8, at which the new compound is fairly stable. In due course the new substance was isolated and shown to be a *phosphocreatine*, to which the

name of *phosphagen* and the following formula have been assigned:



Later investigations showed that this compound is present in the striated, smooth and cardiac muscles of all classes of vertebrates, but absent from those of invertebrates (see p. 283 et seq.). In its place, invertebrate muscles contain an analogous derivative of arginine, *phosphoarginine*, with the following structure:



In passing it should be noticed that both phosphagens contain an energy-rich phosphate bond, a feature which turns out to be of great physiological significance. In what follows we shall discuss mainly the creatine compound, but it may be assumed that what goes for this substance is also true of the arginine analogue.

A wave of interest in the new compound soon developed, and within a few years it became known that it plays an important part in the chemistry of muscular contraction. Phosphagen, it was shown, breaks down during activity and is resynthesized during rest, aerobically and anaerobically alike, and, moreover, it breaks down far more rapidly than does glycogen. It was suspected by some that, since the breakdown of phosphagen precedes that of glycogen, it must be the immediate source of contraction energy, the more slowly acting process of glycolysis being used to resynthesize the phosphagen, rather as the lever of an air-gun is used to reset the spring after the trigger has been pulled. But this idea did not find much favour; muscle chemists were still too much wedded to the older lactic acid hypothesis.

In 1930, however, new evidence appeared. Lundsgaard, who was studying the pharmacological properties of iodoacetic acid, observed that in animals dying from iodoacetate poisoning the muscles went into rigor, but that, instead of becoming markedly acid as was to be expected, they actually became faintly alkaline. Closer examination showed that no lactic acid whatever had been produced. This discovery caused a good deal of surprise, since it demonstrated conclusively that muscle can contract without producing any lactic acid at all. Further work on iodoacetate-poisoned muscles showed that phosphagen breaks down when work is done, the amount split being strictly proportional to the amount of energy expended. Further, phosphagen was not resynthesized if an iodoacetate-poisoned muscle was allowed to rest, and, with repeated stimulation, the muscle went into rigor as soon as its stock of phosphagen was exhausted.

By this time it had begun to appear that muscle resembles yeast rather closely in its carbohydrate metabolism. Compounds such as the hexose phosphates could be detected as well in the one as in the other, while that all-important compound adenosine triphosphate was also present in both. Most of the work so far described had been done on intact, isolated muscles, mostly of frogs, kept under anaerobic conditions, but in 1925 Meyerhof published a method for the preparation from muscle of an extract analogous to the yeast juice that had been so valuable in the study of fermentation. The method employed is roughly as follows.

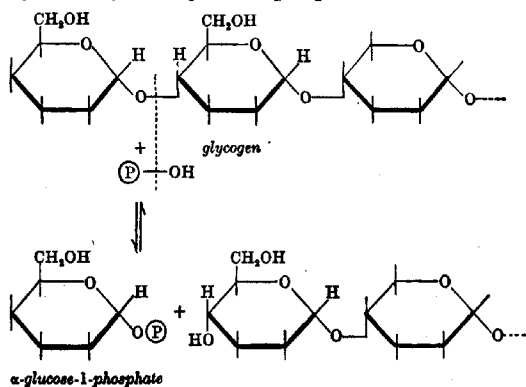
The animal is anaesthetized and cooled to 0° C. The muscles are carefully cut away with the least possible injury, and care is taken to keep them cold. The tissue is put through an ice-cold mincer and allowed to stand for 30–60 min. with ice-water or isotonic KCl. After straining and centrifuging, a rather viscous liquid is obtained and stored in the refrigerator until required.

Extracts prepared in this way contain all the enzymes and coenzymes required for the production of lactic acid from added glycogen, and will also break down creatine phosphate and ATP if these are added. Most of the recent progress in muscle chemistry has been achieved with the aid of extracts of this kind. Dialysis removes the coenzymes, just as it does in the

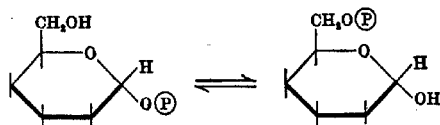
case of yeast juice, and many experiments have been carried out with extracts previously dialysed or treated with fluoride or iodoacetate. One point that should be noticed is that, although these extracts can convert glycogen into lactic acid, they do not respire, so that it is possible to work on extracts in the presence of air instead of having to take elaborate precautions to ensure anaerobiosis, as is necessary when isolated muscles are employed. Whereas the enzymes concerned in glycolysis are soluble and come out into the extract, cytochrome oxidase and certain other enzymes essential for respiration are not soluble but remain attached to the cell debris. Another important feature of these extracts is that, unlike intact muscle, they have no action upon glucose. Intact muscle cells can convert glucose into glycogen with the aid of an enzyme closely resembling the hexokinase of yeast, but this enzyme is insoluble and consequently absent from muscle extracts.

FORMATION OF LACTIC ACID

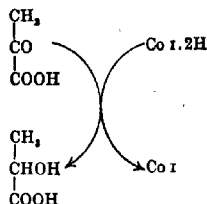
The reactions involved in glycolysis are very similar to those involved in alcoholic fermentation. The first stage in muscle extract consists in the phosphorolysis of glycogen, i.e. splitting of the glycogen molecule by the elements of phosphoric acid. The reaction, which is reversible, is catalysed by *muscle phosphorylase* and yields α -glucose-1-phosphate:



The product is then converted into glucose-6-phosphate by *phosphoglucomutase*:



From this point glycolysis and fermentation follow a common path until pyruvic acid is formed. Here the paths diverge again, for muscle, unlike yeast, does not contain carboxylase. Co-carboxylase is present, but, like Co I and Co II, it collaborates with more enzymes than one, and its presence in muscle is in no way an indication that carboxylase itself is present. In yeast, it will be remembered, pyruvic acid is split into carbon dioxide and acetaldehyde, the latter then functioning as a hydrogen acceptor in the reoxidation of reduced Co I. In muscle extract, however, no carboxylase being present, pyruvic acid itself discharges this function and is reduced to L-(+)-lactic acid, under the influence of *lactic dehydrogenase*:



The overall effect of this reaction sequence is that, on the carbohydrate side, one 6-carbon unit of glycogen yields two molecules of lactic acid. Co I is alternately reduced and reoxidized just as it is in fermentation, but there are differences as far as the ATP/ADP system is concerned. In yeast juice and in muscle extract alike the sequence as a whole leads to the generation of four new energy-rich phosphate bonds for each 6-carbon unit metabolized. In fermentation, two of these new bonds are used to replace those used in the preliminary phosphorylation of the

glucose molecule, so that in this case there is a net gain of two molecules of ATP for each glucose molecule fermented. Muscle extract, however, starts from glycogen, not from glucose, and the first stage in glycolysis consists in the splitting of glycogen by phosphoric acid, not by ATP. The product, glucose-1-phosphate, is then converted into the 6-phosphate, and *only one molecule of ATP has therefore to be used up in the production of each molecule of fructofuranose diphosphate*. Of the four molecules of ATP subsequently produced, only one is required to restore the status quo, so that *in muscle glycolysis there is a net gain of three molecules of ATP for each 6-carbon unit of glycogen metabolized*, as compared with a gain of two molecules for each 6-carbon *glucose* unit metabolized in the case of fermentation.

Calculations show that the conversion of glycogen into lactic acid, under biological conditions, is accompanied by a loss of free energy equivalent to approximately 57,000 cal. for each 6-carbon unit glycolysed. Three new energy-rich phosphate bonds are formed at the same time, so that the amount of energy 'captured' in the form of energy-rich bonds is about 30,000 cal., which corresponds to 50-55 % of the total free energy exchange. This, of course, is in muscle extract, and there are reasons for believing that an even higher proportion of the energy may be 'captured' in the intact muscle cell. This is really a remarkable performance when it is realized that the efficiency of even the most modern superheated steam turbines barely reaches 50 % or thereabouts.

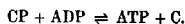
PARTS PLAYED BY ATP AND PHOSPHAGEN

The reactions of glycolysis involve breakdown and resynthesis of ATP, just as do those of fermentation, and the resemblances between the two processes are very striking indeed. There is, however, one very important difference between yeast and muscle, for whereas the latter contains phosphagen, yeast does not.

If an intact, isolated muscle is allowed to contract under anaerobic conditions there is a decrease in the amount of phos-

phagen present, together with corresponding increases in the amounts of free creatine and free inorganic phosphate. This suggests that muscle must contain an enzyme catalysing the hydrolysis of creatine phosphate. This possibility was investigated by Lohmann. If creatine phosphate is added to a preparation of muscle extract it is rapidly split into creatine and inorganic phosphate. But, Lohmann found, there is no hydrolysis of phosphagen added to a dialysed extract, indicating that some dialysable factor is involved. This factor was identified with ADP, and it was then discovered that phosphagen is not hydrolysed, as had formerly been supposed, but that it reacts with ADP, a phosphate radical being transferred, yielding free creatine together with ATP. ATP itself is then split by a powerful adenosine triphosphatase that is present in the extract, ADP is again formed and phosphorylated again at the expense of the phosphagen and so on, until no more phosphagen remains.

Lohmann further discovered that adenosine triphosphatase can be inactivated by prolonged dialysis at 0° C. and, when the enzyme had thus been destroyed, he was able to show that the reaction between creatine phosphate and ADP is freely reversible:



Very little energy change is involved and no inorganic phosphate is set free in this, which is known as the Lohmann reaction. From the existence of this equilibrium system we can make certain deductions concerning the part played by phosphagen in the economy of the muscle: anything that tends to decompose ATP will force the reaction over towards the right and phosphagen will be broken down. If, on the other hand, glycolysis is in progress so that new energy-rich bonds are being generated, fresh ATP will be formed, the reaction will swing towards the left and phosphagen will be resynthesized.

Perhaps the most important feature of Lohmann's work was his discovery that, until some ATP has been broken down to provide ADP, no decomposition of phosphagen can take place: in other words, *the breakdown of ATP must take place even earlier than that of phosphagen*. The breakdown of ATP, in fact, is the

earliest reaction we have so far been able to detect, and hence is the most probable *immediate* source of contraction energy. This suggests that ATP must play some part in contraction, over and above the part it plays in glycolysis.

The probable nature of this additional function was revealed by the work of Engelhart and Lubimova, who found that the adenosine triphosphatase of muscle was inseparable from, and apparently identical with, myosin. Myosin, the contractile protein of muscle, appeared therefore to be identical with the enzyme that catalyses the hydrolysis of ATP and leads to the liberation of the energy of its terminal energy-rich bond. Experiments by D. M. Needham, J. Needham and others have given results which are completely in accordance with the supposition that, when myosin and ATP come into contact, a sudden shortening of the myosin molecules takes place, followed by decomposition of the substrate and a return of the myosin molecules to their normal length. If we accept these results at their face value it follows that the first reaction taking place when a muscle is stimulated consists in the hydrolytic decomposition of ATP by myosin, a process which is attended by the shortening of the myosin molecules and, in some manner which is still quite obscure, the conversion of the energy of the terminal energy-rich bond of the ATP into the mechanical energy of the contraction. More recently a partial separation of ATP-ase activity from myosin has been accomplished¹ but this does not invalidate the conclusion that a very intimate relationship exists between the source of contraction-energy (ATP), the enzyme that catalyses the liberation of that energy (ATP-ase), and the actual contractile material (myosin), whereby the chemical bond-energy of ATP is transformed into the mechanical energy of the contraction itself.

The function of the phosphagen now becomes clear. The muscle contains relatively small amounts of ATP, and these would soon be exhausted but for the fact that the ADP formed by its hydrolysis can be rephosphorylated at the expense of phosphagen, through the Lohmann reaction. This means that

¹ This claim was subsequently withdrawn.

the muscle can go on acting several times longer than it could if no phosphagen was present, for the amount of phosphagen present in a typical striated muscle is considerably greater than that of ATP. These facts are illustrated in Table 24.

TABLE 24. PHOSPHORUS PARTITION IN STRIATED MUSCLE
(mg. P per 100 g.)

Animal	Inorganic-P	Phosphagen-P	Pyro-P	Ratio
				$\frac{(\text{Inorg.} + \text{Phosphagen})\text{-P}}{\text{Available pyro-P}}$
Frog	30	50	30	5.3
Rat	35	80	40	5.7
Rabbit	26	62	40	4.4

Notes. (1) The 'available' pyrophosphate-P is 50% of the total pyrophosphate-P of the ATP present, since only the terminal energy-rich bond is directly available through the activity of adenosine triphosphatase.

(2) The inorganic phosphate found in trichloroacetic extracts of muscle is mainly formed by the breakdown of some of the phosphagen during the extraction. The sum, (inorganic + phosphagen)-P may therefore be taken as an approximation to the true phosphagen-P of the resting muscle.

Phosphagen, then, may be regarded as a reserve of phosphate-bond energy. The energy of its energy-rich bonds is not *immediately* available to the muscle machine but can rapidly be made available by transference to ADP through the Lohmann reaction. In creatine phosphate the free-energy value of the phosphate bond has been estimated at about 10,000 cal. per g.mol., and that of arginine phosphate is about the same.

In all, three ways are known in which ATP can be synthesized from ADP in muscle. The first of these, the Lohmann reaction, is used at the onset of contraction and enables the muscle to keep up a high level of immediately available energy in the form of ATP by drawing upon the stored bond-energy of phosphagen. The second source of supply consists in the new energy-rich bonds generated in the course of glycolysis, and this is the main ultimate source of energy when activity is prolonged. Glycolysis gets under way relatively slowly, however, and phosphagen is used to tide the muscle over the interval between the onset of activity and the establishment of the glycolytic reaction sequence. But there is yet a third possible source of ATP, though this is probably only used when the muscle is *in extremis*. In all the processes mentioned so far, only the terminal energy-

rich bond of ATP is involved. After this has been utilized there remains another such bond in each ADP molecule, but this is not accessible to adenosine triphosphatase. Muscle, however, contains myokinase and this enzyme, acting upon two molecules of ADP, catalyses the transfer of one phosphate radical from one molecule to a second, yielding a molecule of ATP, together with adenylic acid (see p. 298). As has been suggested, this way of producing ATP is probably only used as a last resort, for free adenylic acid is highly toxic and, once formed, rapidly undergoes deamination at the hands of the adenylic deaminase of the muscle to yield inosinic acid. This latter substance cannot replace adenylic acid as a carrier of phosphate, nor does the action of the adenylic acid deaminase appear to be reversible.

CHEMICAL EVENTS IN NORMAL CONTRACTION

We are now in a position to consider the probable course of events in normal muscular contraction. It will be convenient first to consider what takes place during contraction in a muscle previously poisoned with iodoacetate. This drug, it will be recalled, abolishes the activity of triosephosphate dehydrogenase and therefore puts a stop to glycolysis, so that only two sources of ATP are available to the cells.

On the arrival of a nerve impulse, ATP is broken down, giving rise to ADP and inorganic phosphate, furnishing at the same time the contraction energy. The ADP is promptly converted again into ATP at the expense of phosphagen and no change in the ATP content of the muscle can be detected; some phosphagen disappears, however, and is replaced by free creatine and free inorganic phosphate. If repeated stimuli are applied to the muscle, these processes continue until, in the end, no phosphagen remains. As a last resort the myokinase of the muscle is called into play and the last traces of ATP are decomposed, giving, in the end, adenylic acid, which is deaminated. The muscle goes into rigor, and the ammonia produced by the deaminase can be detected in the cells.

In the case of a normal, unpoisoned muscle, glycolysis also comes into the picture. If the muscle is allowed to work

anaerobically the following phenomena can be observed during the *period of anaerobic activity*:

- ATP remains unchanged.
- Phosphagen disappears.
- Free creatine appears.
- Free inorganic phosphate appears.
- Glycogen disappears.
- Lactic acid is formed.

These changes continue as long as the muscle is active. There then follows a short *period of anaerobic recovery* and during this interval, which amounts to about 30 sec., the following further changes take place:

- ATP remains unchanged.
- Phosphagen is resynthesized.
- Free creatine disappears.
- Free inorganic phosphate disappears.
- Glycogen disappears.
- Lactic acid is formed.

Thus glycolysis continues for a short time even after the cessation of muscular activity, and this 'glycolysis of recovery' is attended by resynthesis of phosphagen and a return to the *status quo* of the resting muscle apart, of course, from the conversion of some glycogen into lactic acid.

All these phenomena can be accounted for in terms of the reactions we have considered. While activity lasts, ATP is broken down to provide the energy expended by the muscle. Phosphagen is used up to maintain the level of ATP and a corresponding amount of creatine and inorganic phosphate are set free. The free phosphate is taken up, for the phosphorylation of glycogen in the first instance, and later for the conversion of 3-phosphoglyceraldehyde into the 1:3-diphosphate. This is followed by the generation of new energy-rich bonds in the usual way, the new bonds being transferred to ADP and fresh ATP synthesized. This relieves the drain on the phosphagen stores of the muscle and, indeed, once glycolysis is well established, new energy-rich bonds are usually generated more rapidly than they are expended in the breakdown of ATP. The surplus energy-rich bonds are transferred through ATP to free creatine accordingly,

and phosphagen begins to be resynthesized even while activity is in progress, and it has been shown that during a short period of moderate activity there is at first a steep fall in the phosphagen content of the muscle, followed by a rise to a new, steady level.

During the period of anaerobic recovery, glycolysis continues as long as free inorganic phosphate is available, glycogen accordingly disappearing and lactic acid being formed. ATP is synthesized as long as glycolysis is in progress and, since it is no longer being broken down for energy production, tends to accumulate. The Lohmann reaction therefore goes in reverse and the remaining free creatine is esterified.

GLYCOLYSIS IN TISSUES OTHER THAN MUSCLE

That two types of cells so different in morphology and function as yeast and muscle should make use of practically the same reactions for the breakdown of carbohydrate could hardly have been anticipated. The discovery that such close parallels exist between the two seems to be a hint that the reactions we have been considering may perhaps form a part of the fundamental metabolic equipment of living cells in general, and this does in fact seem highly probable. There is now evidence that tissues other than muscle, tissues such as liver, kidney, brain and so on, make use of reactions which are essentially the same as those employed in muscle. It seems possible that many bacteria and even plants also contain many or most of the enzymes involved in fermentation and in muscle glycolysis, and that ATP is an almost universal go-between which gathers up available energy from carbohydrate sources and stores it up in an immediately accessible form.

The principal differences between different animal tissues from the point of view of their glycolytic mechanisms lie in the amounts of phosphagen they contain (Table 25). The larger and more powerful skeletal muscles usually seem to contain larger amounts of phosphagen, while the slow-acting, smooth muscle of the gastro-intestinal canal, for example, contains only a fraction, amounting to perhaps one-fifth, of the amount present in the average striated muscle. Cardiac muscle also contains rela-

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tively little. The only tissues known to contain phosphagen in concentrations comparable with those of striated muscle are the electric organs of certain fishes, e.g. *Torpedo*. Like striated muscle, these organs are capable of going into activity almost instantaneously and of dissipating very large amounts of energy in very short periods of time. There can be little doubt that the phosphagen mechanism is a device which makes it possible for an organ to go rapidly into action and to do a large amount of work in a short time. Our knowledge of the part it plays in muscle certainly confirms this idea.

TABLE 25. CONCENTRATION OF PHOSPHAGEN IN VARIOUS TISSUES

Organ and animal	(mg. P per 100 g.)			
	Creatine phosphate	Arginine phosphate	Inorganic phosphate	Total phosphagen + inorganic-P
Striated muscle:				
Rabbit	62	.	26	88
Guinea-pig	22	.	58	80
Frog	50	.	30	80
Sea-urchin	11	18	9	38
Scallop	0	42	25	67
Electric tissue:				
Electric ray	37	.	25	62
Cardiac muscle:				
Rat	5	0	31	36
Unstriated muscle:				
Rat (stomach)	3	0	13	16
Sea-cucumber (body wall)	.	28	5	33
Nerve:				
Dog (brain)	12	.	.	12
Rabbit (sciatic)	6	.	9	15
Frog (sciatic)	7	.	10	17
Testis:				
Rabbit	1.4	.	11.6	13
Jensen sarcoma	1.2	.	22	23.2

Phosphagen is also present in spermatozoa in appreciable amounts, and there is some evidence that these cells draw the energy for their locomotion from typical glycolytic processes involving ATP and phosphagen. Traces of phosphagen are found

in nerve, brain and various glandular structures, and it has been suggested that it plays a part in their activity also. Tissues of this kind are usually richly vascularized, however, and it may be that the phosphagen present is associated with the muscular cells that enter into the make-up of the blood vessels.

The phosphagen system apart, glycolysis seems to be an almost universal feature of the activity of living cells, at any rate under anaerobic conditions, and it seems tolerably certain that ATP likewise occurs almost universally. In view of the evidently great importance of these processes, therefore, the reaction-sequence of glycolysis is summarized for reference in Fig. 28, though the part played by phosphagen has been omitted from the scheme for the sake of simplicity. The enzymes and their coenzymes and inhibitors are tabulated in Table 26 for reference.

Emphasis must now be placed upon one feature of the glycolytic reaction sequence that hitherto has been but little mentioned, namely that, as far as the carbohydrate side is concerned, all the reactions involved are reversible. It was believed until very recently that the phosphate-transfer reaction between phospho-*enol*-pyruvic acid and ADP is irreversible, but it has now been shown that ATP can phosphorylate pyruvic acid, given certain conditions, viz. (a) a high concentration of ATP and (b) the presence of potassium ions. One stage on the reverse sequence requires special comment, viz. the dephosphorylation of fructofuranose-1:6-diphosphate to yield the 6-monophosphate. This does not proceed by reaction with ADP, since the sugar ester contains no energy-rich bond such as is required for the phosphorylation of ADP. But dephosphorylation can be carried out hydrolytically with the aid of a phosphatase, and phosphatases are present in most cells and tissues. Hence, as far as the carbohydrate side of glycolysis is concerned it is true to say that glycolysis is a reversible operation.

According to the work of Meyerhof on frog muscle, some 80% of the lactic acid formed during anaerobic activity is reconverted into glycogen in the muscle when the latter is allowed to rest in oxygen. The remaining 20% is oxidized to furnish the energy

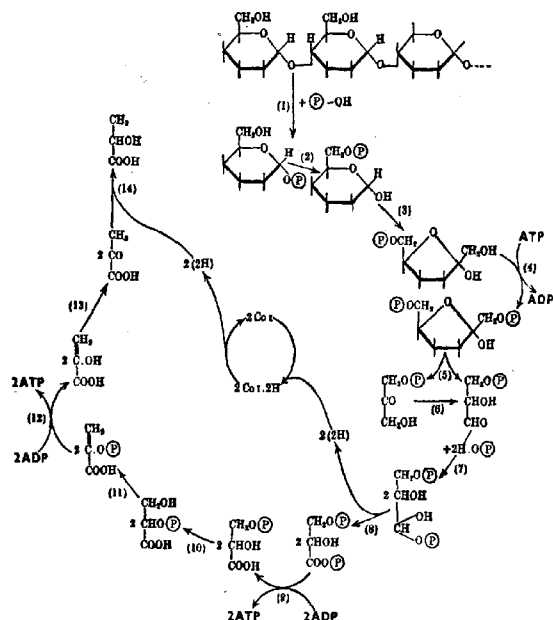


Fig. 28. Scheme to summarize reactions of glycolysis. The part played by phosphagen (where present) is omitted. For names of enzymes, coenzymes and inhibitors, see Table 26.

TABLE 26. GLYCOLYSIS: ENZYMES,
COENZYMES AND INHIBITORS
(see also Fig. 28)

Reaction	Enzyme	Coenzyme	Inhibited by
1	Phosphorylase	$-PO_4H_2$	Dialysis
2	Phosphoglucumutase	.	.
3	Oxoisomerase	.	.
4	Phosphohexokinase	ATP	Dialysis
5	Zymohexase	.	.
6	Phosphotriose isomerase	.	.
7	? Spontaneous	$-PO_4H_2$	Dialysis
8	Triosephosphate dehydrogenase	Co I	Dialysis; $CH_3I.CO_2H$
9	Unnamed phosphokinase	ADP	Dialysis
10	Phosphoglyceromutase	.	.
11	Enolase	Mg ions	NaF
12	Unnamed phosphokinase	ADP	Dialysis
13	? Spontaneous	.	.
14	Lactic dehydrogenase	Co I	Dialysis

required for the resynthesis. While we may take Meyerhof's authority for the belief that this happens in frog muscle, there is little reason to think that it normally takes place in mammalian muscle. As Cori and Cori have shown, any lactic acid formed in the mammalian muscle *in situ* diffuses out and is carried, by way of the blood stream, to the liver. Here it is oxidized to pyruvic acid with the aid of lactic dehydrogenase and Co I, the product being phosphorylated, presumably at the expense of ATP, and built up into liver glycogen by reversal of the glycolytic reaction sequence.

Since it is from the stored glycogen of the liver that muscle glycogen is drawn in the first place, it is to the carbohydrate metabolism of the liver that we must now turn our attention.

THE LIVER: GLYCOGENESIS, GLYCOGENOLYSIS AND GLYCOLYCOGENESIS

The carbohydrate metabolism of animals centres round the processes of glycogenesis and glycogenolysis, i.e. the production and breakdown of glycogen. These processes take place mainly in the liver. Glycogen can be formed from carbohydrate materials, in which case we speak of *glycogenesis* but, as we have already seen, it can also be formed by the liver from non-carbohydrate sources such, for example, as certain amino-acids, glycerol, lactic, pyruvic and propionic acids and many other simple substances. In this case, therefore, we speak of *glycolycolysis*. The term *glycogenolysis* is used to refer to the breakdown of glycogen to glucose, as opposed to the more extensive process of disintegration which we call *glycolysis*.

As far as is known, the carbohydrates of the food do not contribute any materials that are 'essential' in the sense that certain of the amino-acids are essential. Their function is pre-eminently that of furnishing a readily metabolized source of energy. The principal monosaccharide formed by the digestion of an average meal is glucose; other sugars play a relatively small part as a rule; but in the infant mammal, whose sole food carbohydrate is lactose, one-half of the lactose molecule gives rise to galactose on hydrolysis. Other monosaccharides that arise from food

include fructose, formed from sucrose and to some extent from the fructofuranosans.

Pentoses, if injected, are not utilized, but appear largely unchanged in the urine, while disaccharides similarly undergo excretion if injected into the blood stream. Glucose, together with fructose, galactose and the rarer sugar mannose, all lead to the deposition of glycogen in the liver. Glucose itself is phosphorylated by hexokinase at the expense of ATP and the product, glucose-6-monophosphate, is transformed by phosphoglucose-mutase into glucose-1-phosphate, the raw material for the direct synthesis of glycogen by liver phosphorylase. Fructose also can be phosphorylated at the expense of ATP under the influence of hexokinase to yield fructofuranose-6-monophosphate which, in turn, is convertible into glucose-6-phosphate by oxoisomerase and hence, by way of glucose-1-phosphate, gives rise to glycogen. How galactose and mannose are converted into the glucose units of the glycogen to which they give rise is not at all clear, nor do we know how the active mammary gland converts glucose into the galactose radicals present in the lactose which it secretes. But no matter which of the utilizable monosaccharides is administered, the polysaccharide formed in the liver is always glycogen. Different methods of administration can produce glycogens which differ somewhat in chain-length and molecular weight but which otherwise are indistinguishable.

From the standpoint of general metabolism it is important to realize that, even under strictly normal conditions, the liver's capacity to form and to store glycogen is by no means unlimited. Rabbits, for example, can be literally crammed with foods rich in carbohydrate, but a glycogen content of more than 18-20 % in the liver is seldom or never realized. Carbohydrate, if administered in excess of the storage capacity of the liver, gives rise to fat, which is deposited in the fat depots of the body to await metabolism when harder times come.

Glycogen can also be formed in the liver by glyconeogenesis, and many different substances are known to be able to contribute to this process. A usual technique for detecting the formation of carbohydrate from non-carbohydrate sources con-

sists in administering the suspected substances to a diabetic or to a phlorrhizinized animal (see p. 178). Alternatively the substances can be administered to starving animals to see whether or not they give rise to the deposition of glycogen in the liver. We have already seen that the deaminated residues of certain amino-acids can give rise to pyruvate, and this the liver is able to phosphorylate, forming phospho-*enol*-pyruvate which is then convertible into glycogen by reversal of the normal reactions of glycolysis (Fig. 28). Any substance that lies on the glycolytic route from glycogen to pyruvic acid, or which gives rise to any substance lying on this route, can be converted into glycogen, and we can account for all the reactions involved in the conversions of such substances. Thus glycerol, a well-known glucose-former, can in all probability be phosphorylated through the agency of one or other of the tissue phosphokinases to yield glycerol- α -phosphate, which can then be oxidized to phosphoglyceraldehyde by the powerful α -glycerophosphate dehydrogenase which is present in animal tissues generally. Phosphoglyceraldehyde lies directly on the route leading back to glycogen and we can therefore give a reasonable account of the processes which underlie the formation of glycogen from glycerol. In other cases, however, we cannot give such a satisfactory explanation. Propionic acid, for example, is capable of giving rise to glycogen, but we do not know how this takes place, nor do we know how glyoxylic acid, the product of deamination of glycine, is converted into glycogen. Propionic acid is of considerable importance, for it is one of the predominant short-chain fatty acids produced from cellulose when the latter is attacked by the symbiotic micro-organisms of the rumen-contents of the sheep. Similar processes probably go on in other herbivores, and it is likely that the bulk of the carbohydrate produced and laid down in such animals arises, not directly from the carbohydrates of the food, but by glyconeogenesis by way of propionic acid.

Glucose does not lie directly on the pathway of glycolysis and its free interconvertibility with glycogen has therefore to be especially considered. There is now abundant evidence that *glucose must be phosphorylated before it can be stored as glycogen*

or can enter into the glycolytic reaction sequence. This indispensable preliminary phosphorylation is catalysed by hexokinase. Most animal tissues, including muscle, contain enzymes analogous to the hexokinase of yeast, which catalyses the phosphorylation of free glucose at the expense of ATP, yielding glucose-6-monophosphate and hence the 1-phosphate, from which glycogen is synthesized by the tissue phosphorylases. (Muscle extracts do not contain hexokinase and cannot, therefore, act upon free glucose. Presumably the enzyme is either destroyed or inactivated by dilution or else is insoluble and remains on the tissue debris.)

Apart from the liver, which is the central storehouse for glycogen, the muscles contain considerable quantities of this polysaccharide. Other tissues contain only small quantities. These peripheral stores of glycogen are built up mainly and perhaps entirely at the expense of the liver glycogen, by way of the circulating glucose of the blood. The interconnexions are illustrated in Fig. 29. Liver glycogen undergoes phosphorolysis to yield α -glucose-1-phosphate, an ester which is readily dephosphorylated by liver phosphatase to give free glucose. It is generally believed that the blood glucose arises mainly in this way, since the other important glucose ester, glucose-6-phosphate, is relatively slowly attacked by liver phosphatase. The free glucose passes into the blood stream. Other tissues such, for instance, as the muscles, take up this free glucose and phosphorylate it at the expense of ATP by means of their hexokinase. The product, glucose-6-phosphate, is transformed into glucose-1-phosphate by phosphoglucomutase in the usual way and hence, with the aid of the tissue phosphorylase, into glycogen. The latter is then held in readiness for use when the need arises. Essentially similar reactions are involved in the synthesis and breakdown of starch in plant tissues.

The concentration of free glucose in mammalian blood is very finely adjusted at a level of about 100 mg. per 100 ml., the precise concentration varying somewhat from species to species. Any rise in the blood-sugar level is compensated by the deposition of glycogen in the tissues, mainly in the liver, and any fall by the

mobilization of more liver glycogen. Precisely how the blood concentration is controlled is still highly uncertain however, but it is perhaps significant that the liver phosphorylase, which is intimately involved in the process, is powerfully inhibited by free glucose.

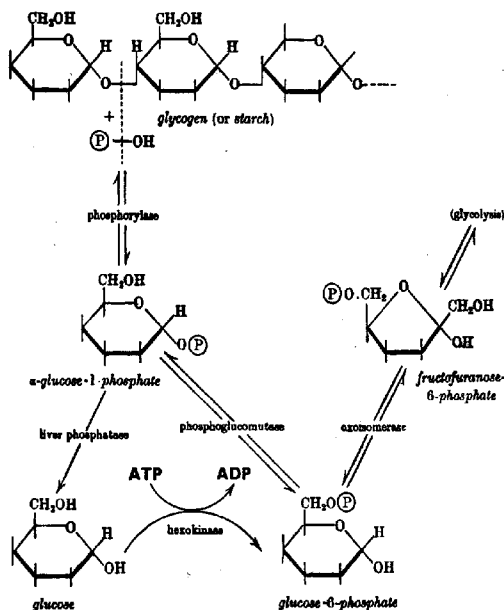


Fig. 29. Relationships between glycogenesis, glycogenolysis and glycolysis. Note the irreversibility of the reaction between glucose and ATP. Free glucose is regenerated from the 6-phosphate by way of the 1-phosphate and liver phosphatase.

The maintenance of the normal level of the blood sugar and the normal storage of glycogen in the liver are profoundly influenced by a number of hormones. Insulin, the internal secretion of the islets of Langerhans, encourages the deposition of glycogen in the liver at the expense of the blood sugar. The diabetogenic hormone of the anterior pituitary body, on the

other hand, encourages the mobilization of glycogen and tends therefore to raise the level of the blood sugar. Clinical diabetes, a condition which is characterized by intense hyperglycaemia and low glycogen storage on the part of the liver, may be due either to insufficiency of insulin secretion or to excessive production of the diabetogenic hormone. It has recently been discovered that certain extracts of the anterior pituitary have a powerful inhibitory action upon hexokinase, an effect that is antagonized by insulin. This discovery goes a long way towards explaining the chemical features of diabetes. If glucose is to be stored and metabolized in the normal manner it must first of all be phosphorylated. A relative preponderance of the hexokinase-inhibitory factor over insulin will lead to suppression of hexokinase activity and this, in turn, to subnormal storage and subnormal metabolism of glucose. The other characteristic features of diabetes (ketosis and ketonuria) are metabolic consequences of the suppression of carbohydrate metabolism (see pp. 390, 409). Although the diabetogenic hormone and the hexokinase-inhibitory factor are both present in extracts of the anterior pituitary there is, as yet, no evidence that the two substances are identical.

Other hormones, notably adrenaline, can also influence the level of the blood sugar, but their effects are usually short-lived and not to be compared with the long-term control exercised by insulin and the diabetogenic hormone. It would profit us little at this stage to go further into the relationships that exist between liver glycogen, blood glucose, and the secretions of the various endocrine organs which have influence upon them, and for exhaustive discussion of these problems the reader is referred to the standard physiological text-books and monographs that have been devoted to them.

CHAPTER XV

AEROBIC METABOLISM OF CARBOHYDRATES

INTRODUCTION

THE bulk of our knowledge of the mechanisms of muscle glycolysis was gained by studies of muscle extracts, which function anaerobically, or else of intact, isolated muscles kept under anaerobic conditions. Now a typical muscle *in situ* enjoys an excellent blood supply, and there is physiological evidence that this blood supply is actually increased when the muscle goes into activity. Analysis of the blood entering and leaving a perfused muscle shows that activity is attended by the utilization of large amounts of oxygen and the formation of correspondingly large quantities of carbon dioxide. Moreover, many muscles contain a special intracellular store of oxygen in the form of muscle oxyhaemoglobin (myoglobin), upon which they can draw for additional oxygen during the interval between the onset of activity and the augmentation of the normal blood supply. In view of these considerations we must enquire whether we have perhaps been led astray by studying muscle metabolism only under anaerobic conditions, and whether anaerobic contraction has any real biological significance at all.

It is characteristic of the anaerobic metabolism of muscle that glycogen is broken down and lactic acid formed. Lactic acid arises by the reduction of pyruvic acid, and its formation provides a mechanism for the reoxidation of reduced Co I. Unless the reduced coenzyme were in some way reoxidized, no further production of phosphoglyceric acid could take place and the generation of new energy-rich bonds for the resynthesis of ATP would come to an end. Under aerobic conditions, however, the reduced coenzyme is rapidly reoxidized through the flavoprotein/cytochrome/cytochrome oxidase reaction chain, and no lactic acid need be produced at all; instead, it might be anticipated, pyruvic acid would accumulate in the muscle. But there is no evidence that pyruvic acid does so accumulate in normal muscle,

nor is there evidence that much pyruvic acid escapes from the muscle into the blood. We are left, therefore, with the conclusion that if pyruvic acid is formed under aerobic conditions, it must be oxidized, and that it is the source of much or all of the carbon dioxide produced by an active muscle. We know that the amount of energy that becomes accessible to an organ or cell that oxidizes its metabolites completely is greatly in excess of that obtained by partial, anaerobic breakdown and, since the muscle is evidently capable of using oxygen and is provided with elaborate devices for providing it with oxygen, it becomes more than ever doubtful whether anaerobic contraction has any biological function, and whether lactic acid is ever formed *in vivo*.

Careful estimations show that small quantities of lactic acid are normally present in the blood, and that the amount is somewhat increased as a result of moderate exercise. After very violent exertion, however, there is a sharp rise in the blood lactic acid, but the level soon begins to fall again as the lactic acid is taken up by the liver and converted into glycogen. A typical curve is shown in Fig. 30, for moderate (*a*) and for strenuous work (*b*). If now we consider the conditions prevailing in an intact muscle *in vivo*, these phenomena can be accounted for. In mild or moderate exercise, oxygen is brought into the cells fast enough to reoxidize the reduced coenzyme as rapidly as it is formed, so that little or no lactic acid is produced, and pyruvic acid, instead of being reduced, is completely oxidized. If now the degree of exertion is increased, glycogen will be more rapidly broken down and Co I proportionately more rapidly reduced. Eventually, with increasing severity of exercise, a point will be reached at which the oxygen supplied by the circulatory apparatus can only just keep pace with the reduction of the coenzyme. But the muscle can work still harder by (*a*) utilizing oxygen as fast as it is made available by the circulatory system, and (*b*) reoxidizing any coenzyme that still remains in the reduced condition by using the anaerobic device of lactic acid formation. In the case of an antelope running for its life from a pursuing lion, the ability to use its muscles anaerobically, above and beyond the limits set by the efficiency of its circulatory apparatus,

may allow of the extra turn of speed that saves the antelope's life: but the lion, unfortunately, can make use of the same trick!

As has been pointed out already, when lactic acid is formed a good deal of potentially available energy remains in the lactic acid molecules, so that anaerobic metabolism is very inefficient from the point of view of the muscle. But this residual energy is not lost to the organism, for the lactic acid rapidly escapes

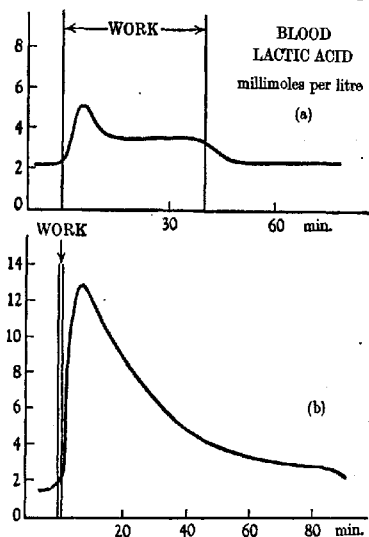


Fig. 30. Changes of blood lactic acid during and after exercise. (Modified after Lipmann, from data by Bang.) (a) moderate work, (b) strenuous work.

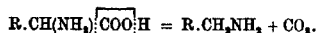
from the muscle into the blood and is transported to that maid-of-all-work, the liver, which proceeds to transform it again into glycogen.

These considerations raise a host of new problems. We must enquire into the mechanisms whereby pyruvic acid is oxidized and into the origin of the respiratory carbon dioxide which is so characteristic an end-product of aerobic, as opposed to anaerobic, metabolism.

ORIGIN OF RESPIRATORY CARBON DIOXIDE

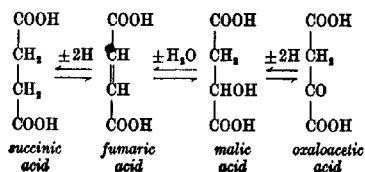
Carbon dioxide can be formed anaerobically as well as aerobically; indeed, it is one of the two chief products of alcoholic fermentation. It is formed in this case by the action of carboxylase and co-carboxylase upon pyruvic acid. Animal tissues, however, contain no carboxylase, although co-carboxylase is present among them, and we must therefore look for other possible sources of carbon dioxide.

Certain amino-acids are decarboxylated in animal tissues with formation of the corresponding amines, together with carbon dioxide:

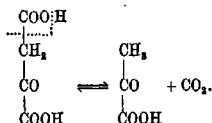


Reactions of this kind take place commonly in bacteria, but they are rare, small-scale processes among animals. Histidine and tyrosine, for example, are deaminated by specific decarboxylases in animals, but the products are immensely powerful pharmacologically and are formed only in traces, so that the yields of carbon dioxide from these sources are comparatively trivial. Not many enzymes of this kind have been detected: those at present known include the histidine and tyrosine decarboxylases already mentioned, together with a specific cysteic acid decarboxylase, but we cannot look to these for the origin of the large volumes of carbon dioxide produced by animal tissues.

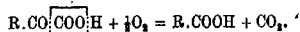
One important potential source of carbon dioxide concerns the dicarboxylic acids, succinic, fumaric, malic and oxaloacetic. These compounds are mutually interconvertible through succinic and malic dehydrogenases together with fumarase, a group of enzymes which appear to be universally distributed:



Under aerobic conditions the tendency in this system is a drift in the direction of oxaloacetic acid. This is an unstable substance which, even *in vitro*, undergoes slow spontaneous decarboxylation in the β -position to give pyruvic acid:



This reaction, which is reversible, is catalysed in liver, though not apparently in muscle, by a specific enzyme known as β -carboxylase. It is interesting as an atypical process, for the usual fate of α -keto-acids is that they undergo *oxidative decarboxylation* at the α -carbon atom:

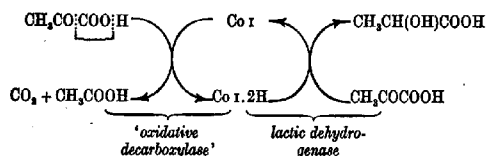


This latter reaction appears to be irreversible.

Two points may be emphasized in connexion with this, which is believed to be a major source of respiratory carbon dioxide. First, it is essentially an oxidative process and therefore differs sharply from the 'straight' decarboxylations catalysed by carboxylase and by the amino-acid decarboxylases. The second important feature is that this reaction, though general for α -keto-acids, does not extend to β -keto-acids.

Although oxidative decarboxylation differs in most respects from the 'straight' decarboxylation catalysed by carboxylase, it resembles it closely in one important particular, namely that it requires the participation of co-carboxylase. This substance is identical with the diphosphate of vitamin B₁ and is often referred to as diphosphothiamine or aneurin diphosphate. The relationship between the oxidation of pyruvic acid and diphosphothiamine has been brought out very clearly indeed by the work of Peters on the brain tissue of B₁-deficient pigeons. Normal brain tissue metabolizes little but carbohydrate, which it oxidizes completely. Peters found, however, that the brain of pigeons deprived of thiamine carries the breakdown of glucose as far as pyruvic acid, but can go no further. If a little thiamine is added

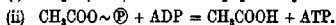
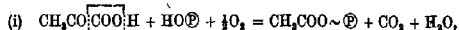
it rapidly undergoes phosphorylation in the tissue, yielding co-carboxylase, and the pyruvic acid begins to disappear. Under aerobic conditions it is completely oxidized, but under anaerobic conditions one molecule of pyruvate can be oxidatively decarboxylated, yielding acetic acid and CO_2 , while a second molecule is reduced to lactate:



Several important deductions can be made from these observations. They show in the first place that diphosphothiamine is indispensable for oxidative decarboxylation, and it may be mentioned in passing that the presence of pyruvic acid in the blood and its excretion in the urine is a regular feature of advanced vitamin B_1 deficiency in animals. Peters's observation that the oxidative decarboxylation of pyruvate is associated with the formation of small amounts of lactate probably indicates that Co I must be involved, since the lactic dehydrogenase that catalyses the reduction requires the collaboration of Co I, and it is known that such a system can only couple with one requiring the same coenzyme. Finally, since the reaction still takes place in an oxidative manner, even under anaerobic conditions, Peters's work serves to emphasize the essentially *oxidative* nature of this particular type of decarboxylation.

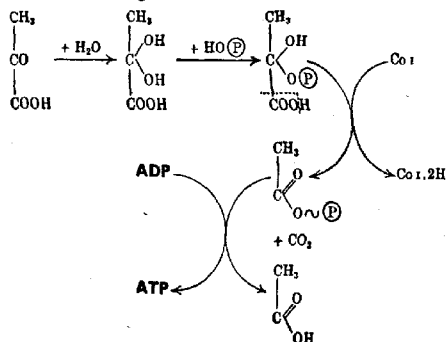
The most complete study of the oxidative decarboxylation of pyruvic acid we owe to Lipmann, who examined the process with the aid of enzymes prepared from a micro-organism, *B. delbrückii*. Lipmann observed that, in crude extracts of this organism, the oxidative decarboxylation of pyruvate was attended by the disappearance of inorganic phosphate from the mixture and by a simultaneous synthesis of ATP. Suspecting therefore that a phosphorylated intermediate of some kind must be involved he went further, and was able to analyse the

process into two distinct stages which may be represented as follows:



The intermediate body, variously known as phosphoacetic acid or acetyl phosphate, was later isolated in the form of its silver salt. Further work with partially purified enzyme preparations showed that the process as a whole requires the presence of a considerable group of small-molecular materials, viz. *diphosphothiamine*, *CoI*, *ADP*, *inorganic phosphate* and *magnesium ions*. The last of these requirements might perhaps have been predicted, since Mg^{++} is necessary whenever diphosphothiamine is required: indeed, magnesium-deficiency leads to symptoms of vitamin B_1 deficiency in experimental animals. Substantially the same observations have been made in studies of oxidative decarboxylation in other cells and tissues, and it now seems highly probable that the mechanisms discovered by Lipmann in his bacteria are essentially the same as those employed by a considerable diversity of cells of other kinds.

We do not yet know the precise details of the intermediate reactions but, bearing in mind the close parallel between the conditions required for oxidative decarboxylation on the one hand and for the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid on the other (p. 316), it seems reasonable to postulate the following reactions:



This scheme accounts for the observed disappearance of inorganic phosphate, for the intermediate formation of acetyl phosphate and for the synthesis of ATP. It accounts also for the fact that ADP and Co I are required, but gives no account of the participation of diphosphothiamine. Since the latter is also required in the 'straight' decarboxylation catalysed by carboxylase, it is perhaps reasonable to suppose that it is specifically concerned with the removal of carbon dioxide from some intermediate reaction complex.

That oxidative decarboxylation leads to the generation of new energy-rich bonds shows that it must be an important process from the point of view of energy production and, as we shall see, it is certainly important as a source of respiratory carbon dioxide. The process appears to be irreversible and is strongly inhibited by arsenite.

STATUS OF PYRUVIC ACID

It will be clear from what has gone before that pyruvic acid occupies a central position in the metabolism of carbohydrates. It can be formed either from glucose (after phosphorylation) or from glycogen (after phosphorolysis) by the normal reactions of glycolysis. By reversal of these processes it can contribute to the synthetic formation of carbohydrate. It constitutes, moreover, a link between the metabolism of carbohydrates and that of proteins, for it can arise more or less directly from the products of deamination of a number of amino-acids. These are only a few of the processes into which pyruvic acid enters, and a summary of the most important of its known origins and fates is presented in Fig. 31.

Under anaerobic conditions pyruvic acid can be broken down by 'straight' decarboxylation, as it is in yeast, or it may undergo reduction to lactic acid, as it does in muscle. If aerobic conditions prevail it can be completely oxidized, as in muscle, kidney, brain and other tissues, or it may be oxidatively decarboxylated to yield acetic acid. Since the latter is a potential source of fat, pyruvic acid may be regarded as a link between the metabolism of carbohydrates and that of fat, as well as that of proteins.

We can give a tolerably satisfactory account of many of the processes listed in Fig. 31. But pyruvic acid can give rise, when incubated with liver tissue under suitable conditions, to citric, α -ketoglutaric, succinic, fumaric and oxaloacetic acids among

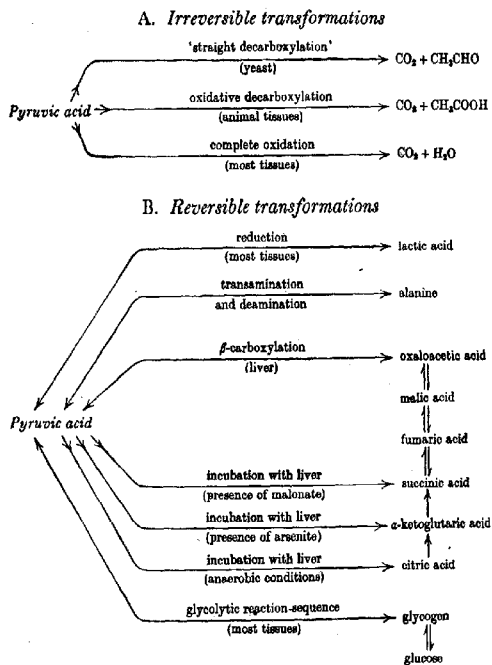
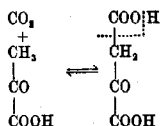


Fig. 31. Formation and fate of pyruvic acid. Note that the synthesis and breakdown, e.g. of succinic acid, do not necessarily follow the same route.

other compounds, and there is evidence that carbon dioxide is 'fixed' when these synthetic reactions take place. Radioactive samples of α -ketoglutarate, for example, have been obtained by the incubation of liver tissue with pyruvate in the presence of radioactive carbon dioxide. This is believed to be due in the

first instance to the β -carboxylation of pyruvic acid, a reaction which is catalysed by the β -carboxylase of liver:



The responsible enzyme was first detected in bacteria, from which it has since been isolated, and there is now good evidence for its occurrence in mammalian liver. It appears, however, to be absent from muscle.

Oxaloacetic acid itself is a very reactive compound under biological conditions, and attempts to demonstrate the formation of its isotopic form in liver provided with pyruvate and isotopic carbon dioxide have not so far proved fruitful. Other substances to which oxaloacetic acid itself gives rise (α -ketoglutarate, citrate) have, however, been isolated and shown to contain the isotopic carbon.

AEROBIC METABOLISM OF CARBOHYDRATES

We know of no oxidizing enzyme or enzymes capable of catalysing directly the complete oxidation of glucose or glycogen. The liver contains glucose dehydrogenases (p. 129) which catalyse the oxidation of glucose to the corresponding gluconic acid, while there occurs in the red blood cells and elsewhere a hexose-monophosphate dehydrogenase (p. 131) which catalyses the oxidation of glucose-6-monophosphate to 6-phosphogluconic acid. We know of the existence in yeast of a further enzyme that can remove one carbon atom from 6-phosphogluconic acid to give 5-phosphoarabonic acid, but there is no evidence for any direct oxidative degradation more extensive than this.

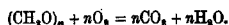
Other sugar acids, the uronic acids, also occur biologically. In particular, glucuronic acid is used by mammals for the detoxication of phenolic and other substances, but there is no evidence that it arises from glucose: indeed, such evidence as we have suggests that it does not.

There is, then, little reason to believe that the 6-carbon units of glucose undergo direct oxidation in the body, and it must therefore be supposed that they are broken into smaller fragments prior to oxidation. We know of at least one way in which such a splitting can be accomplished, viz. by preliminary phosphorylation, followed by the action of zymohehexase, i.e. by glycolysis. This leads, in the end, to the formation of pyruvate, which is known to be completely oxidizable by most tissues when conditions are fully aerobic. We may tentatively visualize the complete oxidation of glucose and glycogen as proceeding by way of the usual glycolytic reactions, yielding pyruvic acid in the first instance, followed by the total oxidation of pyruvic acid itself.

The muscles are the biggest consumers of carbohydrate and it is natural therefore that most of the early work on the aerobic metabolism of carbohydrates should have been done with muscle tissue. Muscle extracts cannot be used for this purpose because, as will be remembered, they do not respire. This fortunate circumstance made it possible to obtain a clear picture of anaerobic glycolysis before the more complex operations of aerobic glycolysis plus oxidation were investigated.

The foundations of our present knowledge of aerobic metabolism were laid by Szent-Györgyi, using minced pigeon-breast muscle as his material. This is a very active muscle and one which contains a good deal of muscle haemoglobin so that, in the minced form, it can keep itself well supplied with oxygen. Indeed, Szent-Györgyi found, minced pigeon-breast muscle respire very actively and produces little or no lactic acid. He studied the rate of respiration of his preparations under a variety of experimental conditions and made the following fundamental observations. The rate of respiration is very high at first but falls off slowly with time. The fall in the rate of respiration is paralleled by the rate at which succinate disappears from the mince, and the respiratory rate of a failing preparation can be restored to its original high level by the addition of catalytic amounts of succinate or fumarate. For each volume of oxygen consumed by the tissue an equal volume of carbon dioxide is formed, indicating

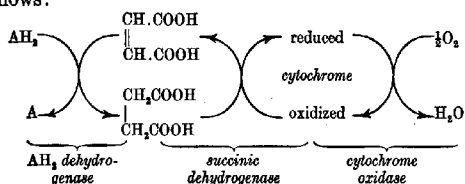
that the material undergoing oxidation is carbohydrate of some kind:



(The ratio carbon dioxide produced/oxygen consumed is called the respiratory quotient and, in theory, is characteristic of the kind of fuel being metabolized: for carbohydrate oxidation R.Q. = 1, while for fat and protein the values are 0.7 and 0.8 respectively.)

From these observations Szent-Györgyi concluded that the oxidation of carbohydrate is in some way catalysed by succinate and fumarate and, since it was known that these two acids are interconvertible through the activity of succinic dehydrogenase, he concluded that this enzyme must be intimately concerned in the oxidation of carbohydrate materials. This led to another important discovery. It had already been established that malonate is a powerful, competitive inhibitor of succinic dehydrogenase, and Szent-Györgyi was able to show that malonate prevents the catalytic effect of succinate and fumarate in failing preparations and, moreover, has a powerfully depressant action upon the respiration of the fresh mince.

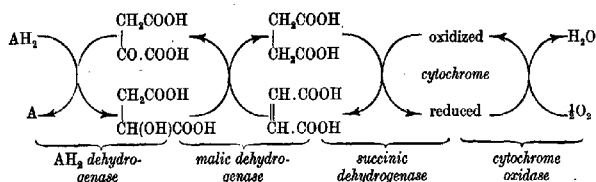
To account for these observations, Szent-Györgyi brought forward the suggestion that succinate and fumarate act together as a carrier system for hydrogen removed from carbohydrate materials of some kind. If we represent these unknown substances by AH_2 , his hypothesis can be schematically represented as follows:



In this system, succinic dehydrogenase would have to work 'backwards' as well as 'forwards' but, as was already known, dehydrogenases in general are capable of acting reversibly, and in this, as well as in its general aspects, the scheme was consistent

with the contemporary knowledge of biological oxidations. Moreover, in this system, everything depends upon succinic dehydrogenase, so that the dire effects of malonate on respiring muscle tissue are readily explained. In the absence of any positive clue to the identity of 'AH₂', Szent-Györgyi suggested that this might be triosephosphate or, perhaps, pyruvate.

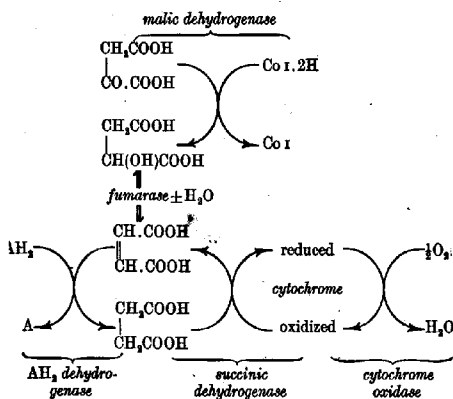
These results stimulated other investigators to study other materials and it soon became clear that the Szent-Györgyi system must be as widely distributed in living tissues as are cytochrome and cytochrome oxidase, for cells and tissues of many kinds were found to behave in the same manner towards succinate, fumarate and the inhibitory substance malonate. Presently it was discovered by Szent-Györgyi himself that two other C₄-dicarboxylic acids act in the same way as succinate and fumarate, viz. malate and oxaloacetate, and he sought to explain these observations by the interpolation of another cyclical carrier stage in his original reaction sequence:



In this way the catalytic action of malate and oxaloacetate could readily be explained, together with the fact that their action, like that of succinate and fumarate, is abolished by malonate.

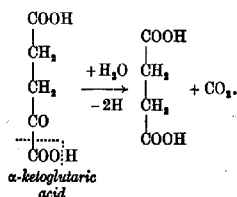
This revised scheme was less fortunate than its predecessor for, when the system was reconstructed from purified components by Green and his collaborators, it was found that the succinic system, which requires no coenzyme, fails to couple with the malic system, which requires Co I for activity. Szent-Györgyi was therefore thrown back upon his original hypothesis, the effects of malate and oxaloacetate being explained by the fact that malate and fumarate are interconvertible through the action of fumarase. Oxaloacetate and malate are themselves intercon-

vertible through malic dehydrogenase and could thus 'feed' the catalytic cycle involving fumarate and succinate:



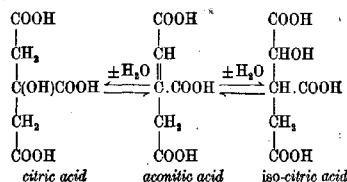
Shortly afterwards Krebs announced the discovery that, in addition to succinate, fumarate, malate and oxaloacetate, α -ketoglutaric and citric acids also act catalytically on the respiration of minced muscle, and that their effects too are inhibited by malonate.

The behaviour of α -ketoglutarate could be explained readily enough, for it was already known that, being an α -keto-acid, α -ketoglutaric undergoes oxidative decarboxylation to yield succinic acid, and thus leads directly to Szent-Györgyi's catalytic cycle:



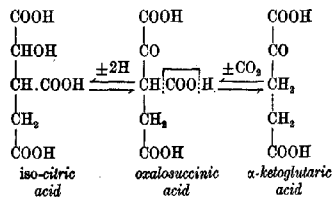
The behaviour of citric acid too was explicable in terms of the widely distributed citric dehydrogenase. This enzyme was

believed to convert citric into α -ketoglutaric acid, from which succinic acid can then be formed. If, however, the formulae of citric and α -ketoglutaric acids are compared, it will be noticed that whereas the ketonic oxygen of the latter is in the α -position, citric acid contains a β -oxygen atom, so that the direct conversion of citrate into α -ketoglutarate seemed very improbable. This phenomenon was explained, however, by the demonstration by Martius and Knoop of a new enzyme, aconitase, which catalyses the conversion of citric into *iso*-citric acid through a common intermediary in the form of aconitic acid:



Reinvestigation of the old citric dehydrogenase now showed that it is specific for *iso*-citric acid, and has no action upon citric acid itself except in the presence of aconitase, with which the early preparations had been contaminated.

iso-Citric dehydrogenase, which collaborates with Co II, catalyses the dehydrogenation of *iso*-citric acid to yield oxalosuccinic (α -keto- β -carboxyglutaric) acid. This compound then, under the influence of a specific oxalosuccinic decarboxylase, loses carbon dioxide and gives rise to α -ketoglutaric acid:



The product can then be oxidatively decarboxylated to yield succinic acid, and thus leads to one of the primary catalysts of the Szent-Györgyi system.

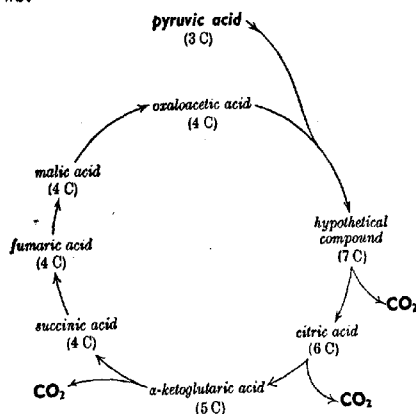
It became possible, therefore, to trace out metabolic connexions between all the substances known to act catalytically on the oxidation of carbohydrates by minced pigeon-breast muscle and by many other kinds of cells and tissues. Their action was explained by their convertibility into succinate and fumarate, the two primary catalysts which, according to Szent-Györgyi, function as a hydrogen-carrying system. The effect of malonate was explained because malonate specifically inhibits the succinic dehydrogenase, upon which the carrier activity of the succinate-fumarate system depends. Thus all the phenomena observed in Szent-Györgyi's original experiments, together with a number of later observations, could be accounted for, apart only from the slow decline that takes place in the respiration of minced pigeon-breast muscle. Even this last phenomenon can now be accounted for, however, by the spontaneous β -decarboxylation of oxaloacetate which takes place under physiological conditions, yielding pyruvate, which does not act catalytically. Oxaloacetate and the other catalytic substances therefore drain slowly away and, as their concentrations decline, the rate of respiration of the preparation falls off.

In passing it should be noticed that the reaction chain leading from α -ketoglutaric, through succinic, fumaric, malic and oxaloacetic acids to pyruvic acid is an important link between carbohydrate metabolism and that of proteins, for α -ketoglutaric, oxaloacetic and pyruvic acids respectively are formed by the deamination of three of the non-essential amino-acids, viz. glutamic acid, aspartic acid and alanine.

Before going on to consider more recent developments the reader will do well to study Fig. 32 (p. 370), in which the reactions just discussed are collected together in schematic form and the compounds shown in relation to some other metabolic products.

The whole picture took on an entirely new aspect with the suggestion by Krebs that pyruvic and oxaloacetic acids might react together to form a 7-carbon compound, from which citrate (6C), α -ketoglutarate (5C), and the 4-C dicarboxylic acids were then re-formed. What had formerly been considered simply as a chain or series of reactions now became in effect a cycle.

In its simplest form this hypothetical scheme can be written as follows:



Pyruvic acid, with its three carbon atoms, enters this cycle by condensing with oxaloacetic acid. With each turn of the wheel one pyruvate molecule enters, three molecules of carbon dioxide are produced, and oxaloacetate is regenerated to take up a further molecule of pyruvate. Since a single molecule of oxaloacetate can be used over and over again in the oxidation of a (theoretically) unlimited number of molecules of pyruvate, it follows that oxaloacetate, or any substance lying on the cycle, can act catalytically in the oxidation of pyruvate to carbon dioxide. The mechanisms postulated involve succinate, fumarate, malate, oxaloacetate, α -ketoglutarate and citrate, all of which are known to act catalytically on the oxidative breakdown of carbohydrate. Succinic dehydrogenase is directly involved in the cycle, oxidizing succinate to fumarate, so that the effect of malonate on the oxidation of carbohydrate is readily explained, while the slow failure of respiration in minced muscle preparations can again be explained in terms of a slow breakdown of oxaloacetate to pyruvate. Krebs's hypothesis therefore goes much further than that of Szent-Györgyi. It accounts at once for the catalytic activity

of all the di- and tricarboxylic acids, it explains the malonate effect and, above all, it accounts for the complete oxidation of pyruvate, a known and important intermediate in the oxidation of carbohydrate, to carbon dioxide. Glucose and glycogen, we

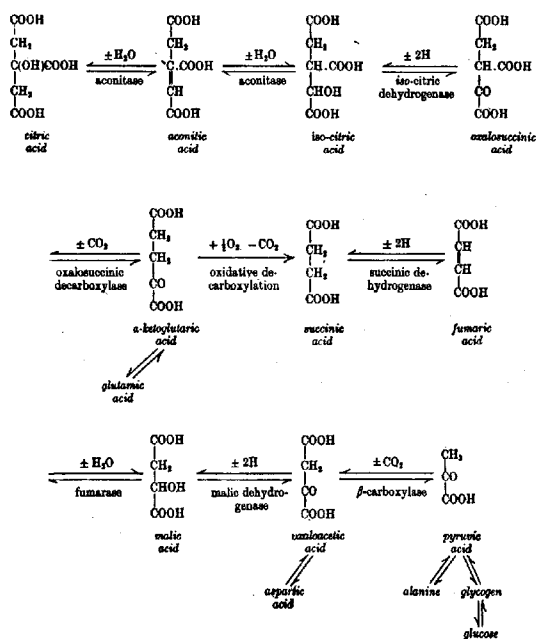


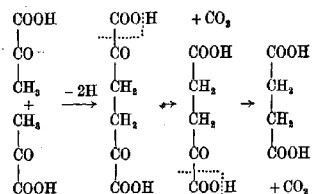
Fig. 32. Summary of reactions leading from citrate to pyruvate, showing some metabolic interrelationships.

know, can be metabolically converted into pyruvate, and Krebs's scheme, taken together with the known reactions of glycolysis, can account therefore for the complete oxidation of glucose and glycogen. Szent-Györgyi's scheme, by contrast, could only account for the dehydrogenation of the unidentified carbohydrate intermediate which we have described as ' AH_2 '.

Krebs himself supplied the first evidence in favour of his

'citric acid cycle'. Pyruvate and oxaloacetate were incubated together with minced muscle under strictly anaerobic conditions. By working anaerobically it was expected that, as the cycle is essentially an aerobic system, some product would accumulate instead of being oxidized if, in fact, pyruvate and oxaloacetate do condense together. After incubation, Krebs was able to demonstrate the formation of substantial amounts of citric acid. Other workers have since repeated and confirmed Krebs's observations on a variety of tissues, and there cannot be much doubt that some reaction involving pyruvate and oxaloacetate does indeed take place.

Other significant experiments have been carried out on liver tissue. If pyruvate is incubated with liver tissue it is possible to demonstrate the formation of considerable yields of succinic acid, together with smaller quantities of α -ketoglutaric acid. This had been known to take place some years earlier, and the synthesis had formerly been explained by the supposition that two molecules of pyruvate react together to give a di- α -keto-acid. This, after oxidative decarboxylation at one end of the chain, would yield α -ketoglutaric acid and, after a second oxidative decarboxylation, succinic acid would be formed:

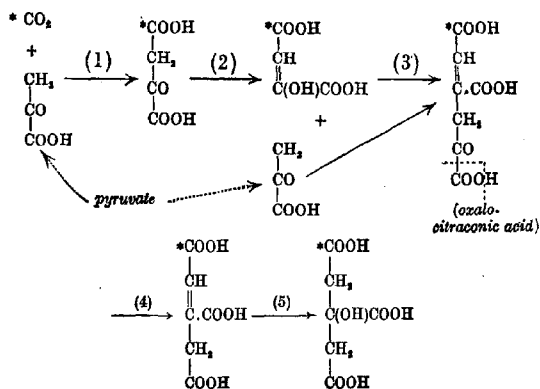


No experimental evidence has ever been found for the initial oxidative reaction, however, though the remaining stages are likely enough. The discovery of β -carboxylation suggested another possible route for the synthesis of succinate from pyruvate, since the latter, by β -carboxylation, yields oxaloacetic acid, from which succinic acid can be formed by the reversed actions of malic and succinic dehydrogenases, together with that of fumarase. In this case, however, the formation of α -ketoglutaric acid

is left unexplained. Krebs pointed out that if this latter route were followed it should, since it involves succinic dehydrogenase, be inhibited by malonate. He therefore incubated pyruvate with liver tissue in the presence of malonate and found that large yields of succinate were still obtained. Hence the synthesis must proceed by some route that does not involve succinic dehydrogenase. This, of course, is entirely in keeping with Krebs's cyclical system, and the evidence was further strengthened by the later demonstration that, in addition to succinate, substantial amounts of α -ketoglutarate and traces of citrate are formed at the same time.

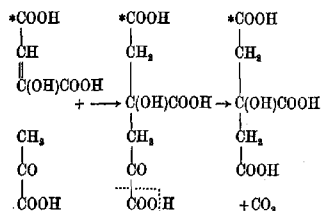
Perhaps the most illuminating evidence regarding the Krebs cycle was that obtained by Wood and Evans and their co-workers, employing radioactive carbon as a 'tracer'. If pyruvate is incubated with liver containing radioactive carbon dioxide, radioactive α -ketoglutarate can subsequently be isolated. This again argues in favour of the cycle, and more conclusive evidence could be obtained by finding out precisely where, in the α -ketoglutarate molecule, the radioactive carbon was located.

If citric acid lies on the cycle, any citric acid formed from pyruvate and radioactive carbon dioxide would itself be radioactive. Its synthesis can be represented, following Krebs's formulation, in the following manner, using asterisks to denote radioactive carbon atoms:



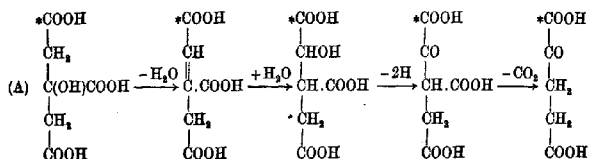
The reactions involved here are: (1) direct addition of carbon dioxide to pyruvic acid, catalysed by β -carboxylase (which is present in liver), (2) enolization of the product, followed by (3) condensation of the *enol*-oxaloacetate with a second molecule of pyruvate to yield oxalocitraconic acid. The next reaction (4) consists in a typical oxidative decarboxylation giving rise to aconitic acid which then (5) takes up water to give citric acid. It will be seen that the radioactive carbon atom is terminally situated.

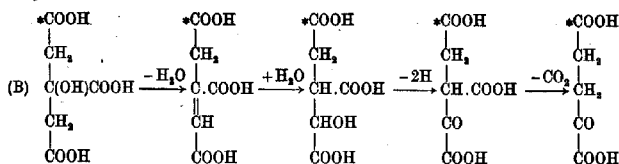
Alternatively, it might be supposed that pyruvate and oxaloacetate form an addition compound instead of condensing together, a process which would lead more directly to the formation of citric acid:



In this case again the radioactive carbon atom would be terminally situated in the resulting citric acid.

Now in its subsequent conversion into α -ketoglutaric acid, citric acid would have to go through aconitic and *iso*-citric acids and, being a symmetrical substance, might react in either of two equally probable ways. One route (A) would lead to an α -ketoglutarate with radioactive carbon in its α -carboxyl group and the other (B) to an α -ketoglutarate of which the γ -carboxyl grouping would contain radioactive carbon:





It follows that if citric acid lies on the cycle, as Krebs believed, the resulting α -ketoglutaric acid should contain radioactive carbon equally distributed between its α - and γ -carboxyl radicals.

α -Ketoglutarate was accordingly isolated from liver tissue previously incubated with pyruvate and radioactive carbon dioxide, and examined. By decarboxylating the product with permanganate it was shown that all the radioactive carbon was present in the α -carboxyl group, for the carbon dioxide released was radioactive while the residual succinic acid was not. As an additional check, succinic acid also was isolated from the liver preparations and found not to contain radioactive carbon. These facts prove conclusively that citric acid itself cannot lie on the cycle. They show, moreover, that the original reaction between pyruvate and oxaloacetate can hardly be an additive process, since such a reaction would lead almost directly to the production of citric acid which, as we have just seen, cannot itself lie on the cycle. Condensation, on the other hand, would yield oxalocitraconic acid and hence, by oxidative decarboxylation, would give rise to aconitic acid which, in the presence of aconitase, could go as well to *iso*-citric acid as to citric acid itself. While these results show clearly that Krebs was wrong in regarding citrate as lying directly on the catalytic cycle, they do, at the same time, provide a brilliant vindication of the scheme as a whole.

There is now good evidence in favour of practically every step in the cycle, with an exception in the case of the supposed 7-C intermediate. Critical experiments designed to detect the formation of such a compound have failed and, indeed, there has never at any time been evidence that such a substance is formed. If a 7-carbon compound is produced it must lose one carbon

atom, most probably by oxidative decarboxylation, to yield aconitic acid. Now arsenite is known to be a powerful inhibitor of oxidative decarboxylation, and it would therefore be anticipated that the formation of oxalocitraconic acid, the most probable 7-carbon intermediary, would readily be demonstrated by working in the presence of arsenite. Actually, however, experiments carried out in the presence of arsenite have led invariably to the isolation of the 5-carbon compound, α -keto-glutarate, together with traces of citrate, but with no trace of a 7-carbon substance. It seems very unlikely therefore that such a compound is formed.

Probably, therefore, aconitic acid must arise directly, presumably by the condensation of oxaloacetate with some 2-carbon substance, rather than with the 3-carbon compound pyruvic acid. It is known that pyruvate readily undergoes oxidative decarboxylation to yield acetate, probably by way of the highly reactive acetylphosphate. It has also been found that the aerobic oxidation of acetate is inhibited by malonate, a feature which indicates that acetate may be oxidized by way of the catalytic cycle. Recent opinion therefore inclines towards the view that pyruvate first undergoes oxidative decarboxylation to yield acetate, the latter then reacting, perhaps in the form of its phosphate, with oxaloacetate to yield aconitic acid directly. The pyruvate may in fact be said to undergo its oxidative decarboxylation before, rather than after, it condenses with oxaloacetate.

We have travelled some way since the first hypothetical scheme was suggested by Szent-Györgyi, and there is little doubt that many details remain even yet to be established. For the moment, however, it will be convenient to recapitulate and summarize the reactions of the 'tricarboxylic acid cycle' as they are now believed to take place. A schematic summary is presented in Fig. 33 and the individual stages will now be briefly reviewed.

(1) Pyruvic acid undergoes oxidative decarboxylation, yielding acetic acid which (2) undergoes condensation with the *enol*-form of oxaloacetic acid. The enzyme catalysing the condensation may be identical with the 'citrogenase' to which we shall refer again later. The product of this reaction is aconitic

'TRICARBOXYLIC ACID CYCLE'

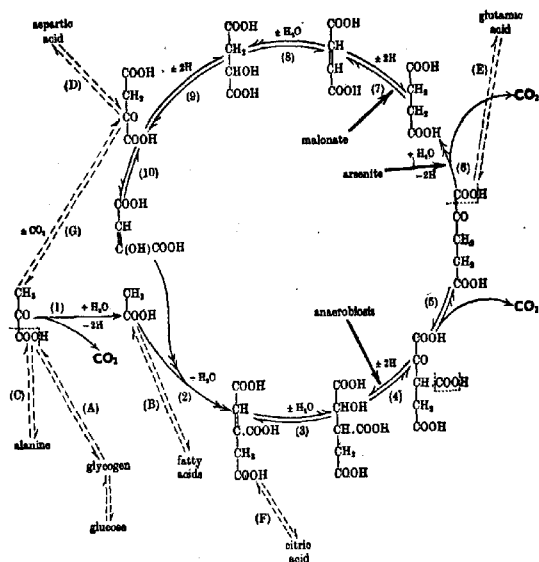


Fig. 33. The 'tricarboxylic acid cycle', showing some important side-reactions and the effects of some inhibitors. For list of enzymes involved see Table 27.

TABLE 27. ENZYMES INVOLVED IN THE TRICARBOXYLIC ACID CYCLE (see Fig. 33)

Reaction no.	Enzymes and coenzymes
1	Oxidative decarboxylation system*
2	† 'Citrogenase'
3	Aconitase
4	iso-Citric dehydrogenase; Co II
5	Oxalosuccinic decarboxylase
6	Oxidative decarboxylation system*
7	Succinic dehydrogenase
8	Fumarase
9	Malic dehydrogenase; Co I
10	Spontaneous
A	Glycolytic system†
B	Unnamed fat-synthesizing enzyme systems
C	Transaminase†
D	Transaminase
E	L-Glutamic dehydrogenase + Co I
F	Aconitase
G	β-Carboxylase

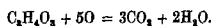
* See p. 359.

† See Figs. 28, 29.

‡ See p. 218.

acid which (3) takes up water and yields *iso*-citric acid under the influence of aconitase. (4) *iso*-Citric acid is dehydrogenated by its dehydrogenase giving rise to oxalosuccinic acid, which, in turn, (5) is decarboxylated by the oxalosuccinic decarboxylase and yields α -ketoglutaric acid. Oxidative decarboxylation of the latter (6) gives rise to succinic acid. This is dehydrogenated (7) to give fumaric acid, which, under the influence of fumarase, is hydrated (8) to yield malic acid. A further dehydrogenation (9) converts this into oxaloacetic acid which (10) enolizes and re-enters the cycle.

Pairs of hydrogen atoms are transferred to the appropriate hydrogen acceptors in reactions (1), (4), (6), (7) and (9), and pass hence through the cytochrome system. In all, therefore, five pairs of H atoms are removed with each turn of the wheel, and three molecules of carbon dioxide are removed at the same time, one molecule of pyruvic acid being used up. These quantities fit the theoretical equation for the complete oxidation of pyruvic acid:



The five atoms of oxygen figured in this equation correspond to the five pairs of hydrogen atoms which, transferred through the cytochrome system, require five atoms of oxygen for their eventual conversion to water.

Although it is certain that most of the reactions are reversible it is probable that the cycle is unidirectional in actual operation, since the reactions involving oxidative decarboxylation (1, 6) are in all probability irreversible. This scheme, every step of which is fairly well established, provides us with an explanation for the complete oxidation of pyruvate itself, of acetate, and of any substance that gives rise to pyruvate or to acetate. It accounts also for the complete oxidation of any substance lying on the cycle, or for the complete oxidation of any substance that gives rise to a compound lying on the cycle. If, for example, a large amount of succinate is added, only a catalytic amount remains in the cycle itself; the remainder passes round the usual reactions until it is converted into oxaloacetate, which breaks down to yield carbon dioxide and pyruvate by β -decarboxyla-

tion. The pyruvate is then oxidatively decarboxylated and oxidized through the cycle. It follows that we have, in this catalytic system, a machine that can accomplish or complete the metabolic oxidation of a great diversity of important primary foodstuffs and their intermediary metabolites.

In addition to the reactions composing the cycle proper, attention may now be paid to a number of important side reactions. Most important among these is the glycolytic reaction sequence (A) which leads from glucose and glycogen to pyruvate and hence to acetate. The cycle allows us now to account for the complete oxidation of glucose, glycogen, and all the known intermediates involved in glycolysis. Furthermore, if, as there is reason to suspect, the breakdown of fatty acids culminates in the production of acetic acid or some highly reactive derivative thereof (B), we can account for the complete oxidation of fat and its intermediary metabolites also.

This ingenious hypothesis also provides a link between the metabolism of carbohydrates and that of proteins. Among the amino-acids that enter into the composition of proteins there are some that are non-essential and can be synthesized in the animal body in seemingly unlimited amounts. Three such are alanine (C), aspartic acid (D) and glutamic acid (E). The corresponding α -keto-acids, pyruvic, oxaloacetic and α -ketoglutaric acids, arise, according to our scheme, as intermediary products in the course of carbohydrate metabolism, and would only require to be aminated or transaminated to yield the amino-acids in question.

Finally, citric acid itself, though not lying on the cycle, can be formed by the action of aconitase upon aconitic acid (F) if the latter tends for any reason to accumulate. This happens if the later reactions of the sequence are inhibited, by anaerobiosis, by arsenite or by malonate. Practically quantitative yields of citrate have been obtained by incubating liver tissue with pyruvic and oxaloacetic acids under strictly anaerobic conditions, suggesting that anaerobiosis completely suppresses the dehydrogenation of *iso*-citric acid (reaction (4)). This may be because the *iso*-citric dehydrogenase requires Co II for its action and, once the available

Co II has been reduced, no system can be found to reoxidize it with appreciable velocity, for relatively few of the known dehydrogenases can collaborate with this coenzyme. The next oxidative reaction in the sequence (6) is an oxidative decarboxylation and, as such, is inhibited by arsenite, so that α -ketoglutaric acid accumulates, but small amounts of citric acid are found at the same time. Malonate inhibits the next reaction (7), in which succinic dehydrogenase is the responsible catalyst, and leads to the accumulation of succinate, together with small amounts of α -ketoglutaric acid and traces of citrate.

Particularly important among the side reactions is the β -carboxylation of pyruvate to yield oxaloacetate (G). In muscle, which contains no β -carboxylase, oxaloacetate drifts slowly out of the system by spontaneous β -decarboxylation, so that the rate of respiration of minced muscle slowly declines. Presumably the maintenance of a high rate of respiration in intact muscle also requires the provision of a constant supply of some one or other of the catalytic dicarboxylic acids. In liver, by contrast with muscle, β -carboxylase is present, and here too oxaloacetate will drift out of the system if the concentration of pyruvate is low. But if pyruvate is suddenly produced, e.g. by the administration of pyruvate to the intact animal or, again, by a sudden burst of glycolysis, oxaloacetate will be formed and the oxidation of pyruvate initiated. Oxidation will continue so long as the concentration of pyruvate remains high, but when it falls again to a low level, oxaloacetate will once more drift out of the system and, as the catalytic carriers fall in concentration, respiration will again slow up. But oxidation will not be the only process tending to reduce the concentration of pyruvate: some will be converted into glycogen, in all probability, some may be oxidatively decarboxylated and give rise to acetic acid and hence to ketone bodies or fat instead of being immediately oxidized, while some may even be transaminated and converted into alanine.

While it is probable in the extreme that a great deal still remains to be discovered about this catalytic cycle and its various side reactions, there can be little doubt of the great and fundamental importance of the system as a whole, both as a clearing-

house for the oxidation of the many products formed on the metabolic lines which converge upon it, and as a meeting-place for the main metabolic pathways of carbohydrate, fat and protein. As has already been pointed out, there is evidence that this cycle, in the form envisaged by Szent-Györgyi, is as widely distributed as are cytochrome and cytochrome oxidase. The cells of animals, plants and micro-organisms appear, with very few exceptions, to contain succinic and malic dehydrogenases, together with fumarase, while *iso*-citric dehydrogenase and aconitase, which together correspond to the citric dehydrogenase originally described by Thunberg, seem likewise to have a very wide occurrence. Again, there is reason to believe that most cells have the ability to accomplish the oxidative decarboxylation of α -keto-acids. It seems not by any means impossible, therefore, that further work will show that the carbohydrate metabolism of living cells in general is organized on the same fundamental lines, glycolysis leading to the formation of pyruvate from glycogen, the pyruvate being metabolized in its turn through the tricarboxylic acid cycle under aerobic conditions, and linking up, as it does in mammalian liver, with the metabolism of protein and of fat.

That we shall find variations on the general, fundamental theme we may be sure, for we have already discovered in the phosphagen of muscular tissues a specialized chemical adaptation which admirably subserves the highly specific functions which muscle is called upon to discharge. Similarly, we may consider that the carboxylase of yeast is a specialization which permits that organism to live on carbohydrate even in the total absence of oxygen. It might, of course, be argued that fermentation is a simpler operation than respiration, and that the simpler must logically be the more primitive but, at the same time, there is evidence that evolutionary advancement and specialization may be attended by the loss of old enzymes, rather than by the acquisition of new, as is the case of the enzymes concerned with purine metabolism (p. 304).

But, beneath all the secondary and specific adaptations that we are likely to meet in the future, there is every reason to

think that we shall discover evidence of the existence of a common metabolic ground-plan to which living cells in general conform.

ENERGETICS OF CARBOHYDRATE OXIDATION

The loss of free energy involved in the glycolytic formation of lactic acid from glycogen under biological conditions is approximately 57,000 calories for each 6-carbon unit of glycogen utilized. For the complete oxidation of glycogen the loss of free energy is about 720,000 calories. Hence the maximum energy-yield for complete oxidation is about twelve times as great as that for glycolysis. If we assume that free energy can be transferred from carbohydrate to ATP with about equal efficiency under aerobic and anaerobic conditions alike, we should expect that, since glycolysis yields 3 (~) per 6-carbon unit, complete oxidation should yield about $3 \times 12 = 36$ (~).

Many experiments have been carried out in order to determine directly the energy-yields of oxidative processes in various tissues. If, for example, dialysed kidney extracts are incubated with succinic acid in the presence of ADP, inorganic phosphate and Mg ions, the system as a whole takes up oxygen, and ATP is synthesized. The amount of ATP formed can be determined and compared with the oxygen consumption of the preparation, and in this way an estimate can be made of the energy-yield of the oxidative metabolism taking place. In one such series of experiments carried out on pigeon-breast muscle, the tissue was previously chopped and exhaustively washed. On incubation in the presence of glucose, with addition of ADP, inorganic phosphate and other essential coenzymes, the yields of ATP recorded ranged from 1 to 4 molecules of ATP for each atom of oxygen consumed. Since the oxidation of one molecule of glucose requires 12 atoms of oxygen, these results indicate an energy-yield of 12-48 (~) per molecule of glucose oxidized.

Now the change in free energy for the oxidation of glucose is about -686,000 calories per g.mol., so that the production of 48 (~), each of which carries about 10,000 cal. of free energy, corresponds to an efficiency of 480/686, or approximately 70 %.

Bearing in mind the possibility that some ATP may have been broken down in the course of incubation we may assume that *at least* 48 (~) can be formed for each molecule of glucose undergoing oxidation and that, given ideal experimental conditions, the yields might conceivably be greater even than this.

We know that of the 50 (~) or thereabouts that are generated when one molecule of glucose is completely oxidized, not more than two are formed during the initial, anaerobic stages that lead to the formation of lactic acid. The residual 48 (~) or thereabouts must therefore arise during the subsequent oxidative stages. Since each molecule of glucose yields two of lactic acid under anaerobic conditions, some 24 (~) must be generated during the oxidation of each molecule of lactate. We know that lactate is dehydrogenated by its dehydrogenase and yields pyruvic acid, and that this pyruvic acid undergoes oxidative degradation through Krebs's catalytic cycle. But of all the reactions involved in this oxidative degradation there are only two in which the formation of energy-rich bonds can at present be accounted for, viz. the oxidative decarboxylation of pyruvic acid itself, and that of α -ketoglutaric acid (reactions (1) and (6)). It follows, therefore, that some twenty-two additional energy-rich bonds must be generated as a result of other reactions on the cycle, and that more than one such bond may perhaps arise as a result of oxidative decarboxylation.

It has been found in experiments with kidney, brain and other tissues that, under suitable conditions, ATP can be synthesized when the tissue is oxidizing added succinate to fumarate, for example, and it seems probable that every dehydrogenation can give rise to new energy-rich bonds. At present we are unable to account for this phenomenon. The only process of oxidation in which we can account for the generation of an energy-rich bond is that in which 3-phosphoglyceraldehyde is oxidized to 3-phosphoglyceric acid (p. 322). On analysis this reaction proved to be considerably more complex than it at first appeared. Three distinct stages occur: 3-phosphoglyceraldehyde takes up inorganic phosphate to form a (hypothetical) 1:3-diphosphoglyceraldehyde, and it is this rather than the monophosphate that is the

actual substrate of dehydrogenation. On dehydrogenation it yields 1:3-diphosphoglyceric acid, in which an energy-rich bond is present, and the third stage in the process consists in the transference of this bond to a molecule of ADP so that ATP is formed. Something similar probably takes place in oxidative decarboxylation, but we know little about the details in this case (see p. 359).

It seems likely, therefore, that even such simple-seeming reactions as the dehydrogenations of succinic and malic acids must, in reality, be considerably more complex than we have so far supposed. When they take place there is a demonstrable synthesis of new energy-rich bonds, and this may mean that inorganic phosphate is incorporated into the substrate, as it is in the case of 3-phosphoglyceraldehyde. If this is indeed true, it is to be expected that succinate, malate and the rest, like phosphoglyceraldehyde, would fail to undergo dehydrogenation if inorganic phosphate were rigidly excluded from the medium, but, in the past, it has been an article of the biochemical faith that enzymic reactions should only be studied in buffered media, and the buffer of choice has usually been one of the phosphate series. Certainly no one has dreamed of trying to exclude every trace of phosphate from his experiments but, had this been done in the past, we might well have known more than we do to-day about the detailed mechanisms of these apparently simple dehydrogenation reactions and, incidentally, about the generation of new energy-rich bonds. There is a field here that holds rich promise for the future.

CHAPTER XVI

THE METABOLISM OF FATS

TRANSPORTATION AND STORAGE OF FATS

ACCORDING to modern opinion a large proportion of the food fat is absorbed in finely emulsified form from the small intestine by way of the lymphatic system and hence, through the thoracic duct, into the blood, where it appears in the form of minute droplets of neutral fat. The remainder undergoes hydrolysis to yield free fatty acids which pass into the portal blood stream directly and so to the liver.

The immediate fate of ingested fatty material has been studied with the aid of deuterated fats, i.e. fats containing heavy hydrogen. Heavy hydrogen gives rise to heavy water when burned and, on account of the numerous and very marked differences between heavy water and 'ordinary' water, heavy hydrogen can readily be detected and estimated by suitable physical methods. Deuterated fats may be prepared by catalytic hydrogenation of unsaturated fats, such as linseed oil, with hydrogen containing a high proportion of heavy hydrogen. Deuterated fat was fed to mice for several days and the animals were then killed. The fats from different parts of the body were extracted from the carcasses, the water formed by their combustion being carefully collected and analysed for heavy water. The results showed quite clearly that the bulk of the deuterium administered had found its way into the depot fats, and only small amounts into the other tissues. We must therefore suppose that the first fate of ingested food fat after absorption is its deposition in the fat depots of the body. Of these the most important are the mesenteries and the intramuscular and subcutaneous connective tissues.

Generally speaking, the kind of fat present in the fat depots of a given animal is fairly characteristic of the species. Beef fat is always much the same in composition, while mutton fat is always characteristically mutton fat. But it is not difficult to

alter the composition of the depot fat considerably by feeding fats of a kind which the animal does not ordinarily consume. If, for instance, a dog is given large amounts of linseed oil it will lay down a softer and much more unsaturated depot fat than is characteristic of dogs as a whole. But it is none the less true that, in the ordinary way, each species lays down its own kind of fat, just as each species lays down its own kind of tissue proteins. The reason for this constancy is only partly covered by the tendency of animals to select a diet which is fairly constant in composition; not all the fat found in the depots is merely food fat that has been transported thither from the alimentary canal, but fat which has been synthesized from non-fat sources. As every stock-breeder knows, animals can be fattened cheaply by feeding them an abundance of carbohydrates, and fat can also be synthesized from protein to some extent. It seems likely that the nature of the fat formed from these sources will depend upon the metabolic make-up of the particular species concerned, different animals starting from much the same raw materials but each manufacturing its own kind of fat.

The synthesis of fats from non-fat sources is particularly important in cattle, sheep and other herbivorous animals, for here cellulose bulks large as a foodstuff. In these animals cellulose is digested by symbiotic micro-organisms which produce from it high yields of short-chain fatty acids, among which acetic and propionic acids predominate, together with some butyric and small amounts of other acids. Acetic and butyric acids are fat-formers, propionic acid yielding glycogen. In animals of this kind, therefore, it is probable that the main reserves of fat and carbohydrate are built up from short-chain fatty acids.

The average animal is capable of laying down almost unlimited amounts of fat and, in point of fact, fat has certain definite theoretical advantages over proteins and carbohydrates as a form of reserve fuel. Fat is far richer in carbon and hydrogen than the other primary foodstuffs, so that there is more combustible material in a gram of fat than in a gram of either protein or carbohydrate. From the point of view of energy, therefore, fat allows the greatest storage per gram of reserve material. If

a gram of each of the three main types of food is burned in a bomb calorimeter the energy produced is approximately as follows:

1 g. protein	5600 cal.
1 g. carbohydrate	4200 cal.
1 g. fat	9300 cal.

Closely bound up with this is the fact that fat, when burned, gives rise to about twice as much water as the other foodstuffs on account of its high content of hydrogen:

1 g. protein	0.41 g. water
1 g. carbohydrate	0.55 g. water
1 g. fat	1.07 g. water

This is an important feature of fat metabolism, especially among terrestrial animals, many of which live under conditions of acute water shortage. In such cases there is commonly a heavy emphasis on the oxidation of fat; in this way the organism is better able to eke out its external supplies of water with metabolic water formed in its own tissues. As an example of this phenomenon we may refer to the developing chick embryo. At laying, the hen's egg is provided with a definite and limited amount of water, an amount which, by itself, would be insufficient to see the embryo through development. But during the 3 weeks of incubation, rather more than 90 % of all the material oxidized by the embryo consists of fat. Again, the mealworm, an insect larva that can live for long periods under the most arid conditions, metabolizes during starvation about $2\frac{1}{2}$ parts of carbohydrate for every part of protein, and no less than 8 parts of fat. The almost legendary ability of the camel to travel for days in the desert without a drink is similarly attributable to heavy fat metabolism with proportionately large-scale production of metabolic water.

Fat which is on its way from the gut to the fat depots is carried mainly in the form of droplets of neutral fat, and a pronounced condition of lipaemia is regularly to be observed after the consumption of a fatty meal. A small proportion of the total fat apparently travels in the form of phospholipoids, for the concentration of phospholipoid materials in the blood shows

a pronounced rise during absorption. When fat is being withdrawn from the depots to be metabolized elsewhere there is no lipaemia, however, and it seems that most of the fat being transported must travel in the form of phospholipoids which, unlike the other lipoids, are appreciably soluble in water. Phospholipoids are normally present in the blood in small quantities, together with a certain amount of cholesterol and cholesterol esters, but neutral fat is only present as such while the condition of post-absorptive lipaemia persists.

The mobilization of depot fat can conveniently be studied in starving animals. After a short period of starvation the glycogen reserves of the liver are used up, and no more glycogen is forthcoming except through glycogenesis. Presumably because of the shortage of carbohydrate, large amounts of fat appear in the liver. This condition, which is known as 'fatty liver', can also be observed in a variety of conditions other than starvation and it seems certain that the accumulation of fat in the liver represents the first step towards its metabolic breakdown.

The fat which appears in a fatty liver arises from the fat depots. As has been pointed out, the administration of deuterated fat to mice leads first to the deposition of most of the heavy hydrogen in the fat depots. But if the animals are allowed to starve for several days before being sacrificed and the body fats then worked up for heavy hydrogen as before, it is found that the fat content of the liver is greater than at the beginning of starvation and, moreover, that the liver fat contains twice or three times as much deuterium as that in the depots. After its absorption, therefore, most of the ingested fat goes first to the fat depots, from which it is withdrawn and transported to the liver as and when the need arises.

The condition of fatty liver can be established by any treatment that tends seriously to diminish the power of the liver to store, produce or metabolize carbohydrate. In diabetes, for instance, the storage powers of the liver are impaired, and fatty liver is one of the features of this disease. Furthermore, in severe cases, the blood may contain three or four times as much fat as normal. In the pseudo-diabetic condition induced by phlorrhizin

the glycogen reserves of the liver are broken down and the glucose thus set free is excreted by way of the urine. Here again fatty liver is to be observed. Small doses of liver poisons such, for instance, as carbon tetrachloride, chloroform, phosphorus and diphtheria toxin, also lead to fatty liver because they disturb the normal functions of the liver with respect to carbohydrate metabolism. In all these cases the establishment of a fatty liver is encouraged by feeding cholesterol and discouraged by the administration of choline or ethanolamine. How the cholesterol effect is produced we do not know, but when choline or ethanolamine is given it seems not unlikely that their arrival at the liver encourages the formation of phospholipoids which, being soluble in water, tend to be carried away.

FUNCTIONS OF FAT: CONSTANT AND VARIABLE ELEMENTS

It will be clear from what has been said that the fat content of the depot tissues and of the liver—and the same is true of other organs—varies very widely with the nutritional condition of the organism. If an animal is allowed to starve for a long time the amount of reserve fat in the body becomes very small, but, even at death from starvation, the tissues still contain a large amount of lipid material which, apparently, forms a part of the structural material of the tissues and is not available for use as fuel. This part of the total body lipoids is referred to as the 'constant element', constant because, being a part of the actual fabric of the organism, it is always present and always must be present. The remainder of the lipoids comprise the 'variable element', so-called because they vary in amount with the nutritional state of the organism and the demands made upon them for energy production.

The contrast between the constant and variable elements can be appreciated by comparing the effects of different doses of liver poisons upon the lipid content of the liver. Small amounts of carbon tetrachloride, for example, lead to the mobilization of fat from the depots and its deposition in the liver. Here we observe the movement of a part of the variable element from

one place to another. If larger doses of the same poison are administered, the liver cells suffer serious damage and we observe the condition known as fatty degeneration. Again the cells are rich in lipid materials, but mainly because the other cell constituents have been broken down and dispersed. Indeed, the lipoids present in fatty degeneration are all that remains of the structural materials of the cell, and represent the constant or structural element of the tissue lipoids.

Fats, then, have two main functions. They act as fuel reserves, and they play an important part in the structure of living tissues. In addition, there are indications that certain fatty acids have special and specific functions, for it is known that rats kept on fat-free diets, or on diets from which particular fatty acids have been carefully removed, become ill and suffer from caudal necrosis, but we shall return presently to consider this condition and its metabolic implications.

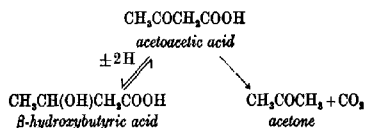
METABOLISM OF FATS

Our knowledge of the mechanisms whereby fats are metabolized is far from being complete and the complexion of the whole subject is changing rapidly at the time this book is being written. The evidence available regarding fat metabolism has in the past been so contradictory and speculation so rife that almost any statement made might be contradicted by as many facts and arguments as could be produced in its support. In what follows, therefore, the author will attempt to give what appears to him to be the most reasonable account of fat metabolism, aware though he is that the picture may have been entirely changed by the time it is printed.

It is generally believed that the liver plays a predominant role in the metabolism of fat, for it is to this organ above all that fat is transported when carbohydrate metabolism is subnormal and an alternative energy source is required. It has usually been assumed, though never proved, that fats are hydrolytically split into glycerol and free fatty acids before any oxidation takes place. This is not entirely an unreasonable supposition, for cells of most kinds seem to be furnished with lipolytic enzymes.

Glycerol, if administered to a diabetic or phlorrhizinized animal, is converted almost quantitatively into glucose. The most probable pathway for this conversion is through phosphoglycerol, phosphoglyceraldehyde and hence, through the normal glycolytic reactions, back to glycogen.

Fatty acids are normally oxidized completely to yield carbon dioxide and water. But in some animals and under certain conditions, fats give rise to large amounts of other compounds, the ketone or acetone bodies. These are acetoacetic acid, together with β -hydroxybutyric acid and small amounts of acetone. It is generally agreed that the parent substance of this group is acetoacetic acid. Under the influence of the widely distributed β -hydroxybutyric dehydrogenase and Co I, acetoacetic acid and β -hydroxybutyric acids are freely interconvertible. Acetone arises from acetoacetic acid by spontaneous decarboxylation, a process which takes place at an appreciable speed under physiological conditions of temperature and pH. The relationships between these three substances can briefly be summarized as follows:



Small quantities of ketone bodies are formed from the ketogenic amino-acids when these are administered to diabetic or phlorrhizinized animals, but that they arise mainly from fat cannot be doubted. Their genesis from fatty acids might be attributable to one of two processes: they might be normal intermediate products of fatty acid metabolism, or they might on the other hand arise as side-products from intermediary metabolites formed during the breakdown of fatty acids.

Traces of ketone bodies can be detected in the blood of normal animals, but the amounts present are greatly increased when there is a heavy emphasis upon fat metabolism. The energy requirements of a typical animal are normally met by metabolizing fat and carbohydrate together, but if, for any reason,

carbohydrate metabolism is subnormal, correspondingly more fat has to be metabolized. Thus ketone bodies accumulate when the food contains disproportionately large amounts of fat. They also accumulate in starvation, once the glycogen reserves of the liver have been exhausted. These reserves can be experimentally drained in fed animals by injecting phlorrhizin, when ketone bodies again make their appearance. They are also formed if the liver's power to store and metabolize glycogen is seriously impaired, as it is in diabetes and in cases of poisoning by chloroform, carbon tetrachloride, phosphorus and other liver poisons.

As Friedmann showed many years ago, there is a similar dependence upon carbohydrate metabolism in isolated, perfused livers. The blood leaving a perfused liver ordinarily contains small amounts of ketone bodies, but the amounts are greatly increased if members of the naturally occurring fatty acids are added to the ingoing blood. The yield of ketone bodies is particularly high when butyric acid is used, but in all cases the yields are higher in livers that are poor in carbohydrate. Similar results have been reported for experiments with liver slices.

Whether we regard the ketone bodies as direct or as secondary products of fatty acid metabolism, it is evident that the organism's ability to oxidize fatty acids is limited in some way, and that the intermediate products fail to be completely oxidized when they are produced in excessive amounts, as they are when carbohydrate metabolism is subnormal. But this is not equally true of all animals. It is more difficult to induce starvation ketosis in rats than in men, for example, while the chick embryo, which metabolizes fat almost to the exclusion of other materials during the period of its incubation, contains not a trace of ketone bodies at the time of hatching. Even among the human species, Eskimos can tolerate diets so rich in fats that the average European on the same diet develops intense ketosis.

So intimate is the relationship between fat metabolism and that of the ketone bodies that any theory of fat metabolism which fails to explain the production of ketone bodies is doomed in advance.

DESATURATION

It has been known for many years that if fatty acids are incubated with liver tissue there is an increase in the iodine number of the sample, indicating that the liver is capable of introducing double bonds into the fatty chain. Leathes pointed out that the liver normally contains rather a high proportion of unsaturated fatty acids, and suggested that fat might be broken down by the introduction of double bonds at more or less arbitrary points along the chain, the latter then being broken into fragments at the 'weak' linkages thus introduced.

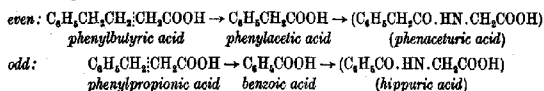
That the liver can dehydrogenate fats is certain, but the notion that double bonds can be introduced *anywhere* in the chain is disproved by the following facts. If rats are given a diet from which all traces of fatty acids with a double bond at C_{12:13} have been carefully removed, they develop a condition known as fat-deficiency disease, characterized by a scaly condition of the tail (caudal necrosis). If the liver were able to introduce double bonds at *any* point in the fatty chain, this condition could hardly exist at all: the disease is curable by the administration of linoleic acid, for example, and this could be made from oleic acid by desaturation in the 12:13 position. This the liver clearly cannot do, at any rate in rats.

Attempts have been made to discover precisely where double bonds can be inserted, by incubating liver tissue with fatty acids already containing double bonds in known positions. When this was done, desaturation still took place except in acids containing a double bond at the $\alpha:\beta$ position. This indicates that desaturation can be accomplished in the $\alpha:\beta$ position. Evidence has been obtained in other ways to show that the liver also contains an enzyme capable of introducing a double bond in the middle (9:10) of the stearic acid chain, giving rise to oleic acid.

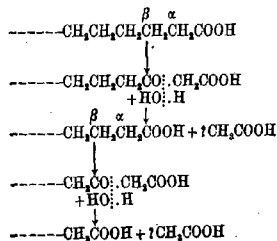
 β -OXIDATION

The introduction of an $\alpha:\beta$ double bond is perhaps the first step in the process known as β -oxidation, first postulated many years ago by Knoop. Knoop prepared a series of ω -phenyl fatty acids,

i.e. fatty acids which had been 'labelled' by the introduction of a phenyl radical at the carbon atom most remote from the carboxyl radical. These acids were administered to dogs, the urine being collected and worked up for substances containing the phenyl group. In every case a phenylated compound was isolated, the products being benzoic and phenylacetic acids respectively. Both acids were eliminated in the form of their glycine conjugates, hippuric and phenaceturic acids, but for our present purposes the conjugation can be neglected. The important feature of Knoop's discoveries was the fact that administration of an acid with an even number of carbon atoms in the side-chain led always and only to the formation of phenylacetic acid, odd-numbered chains giving rise always and only to benzoic acid, e.g.



This must mean that the organism is unable to remove carbon atoms one at a time from the chain: if it could do so, phenylacetic acid would be expected to be converted into benzoic acid, while the administration of either an odd- or an even-numbered chain would be expected to give either benzoic acid alone, or else a mixture of benzoic and phenylacetic acids. Presumably, then, the carbon atoms must be split off in even numbers, most probably in pairs. Knoop accordingly suggested the following scheme to account for the breakdown of the normal fatty chain, the process involved being one of repetitive β -oxidation and hydrolysis:



According to this scheme a typical, long-chain fatty acid would undergo repetitive β -oxidation, yielding a molecule of some 2-carbon substance and a fatty acid with 2 carbons less at each stage. Knoop considered that the 2-carbon substance might be acetic acid, and that this is removed, probably by oxidation, as fast as it is formed. It may be remarked parenthetically that no one has, however, succeeded in isolating or identifying this 2-carbon compound in any quantity, though traces of acetic acid have been isolated from liver in the form of the 2,4-dinitrophenylhydrazide.

Now it happens that the naturally occurring fatty acids are, almost without exception, members of the even-numbered series. Any such acid, by losing two carbon atoms at a time, would pass eventually through the stage of butyric acid which, undergoing β -oxidation in its turn, would give rise to acetoacetic acid. Knoop's scheme could thus account for the production of some acetoacetic acid, especially when fat is being extensively metabolized and, in fact, if butyric acid is given to a diabetic or phlorrhizinized animal, or perfused through an isolated liver, it is largely or even quantitatively converted into acetoacetate and the other ketone bodies.

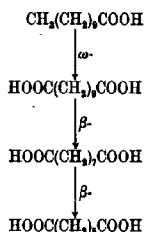
The theory of β -oxidation received much support from Embden's experiments on perfused livers and on diabetic and phlorrhizinized animals. If a fatty acid containing an even number of carbon atoms were perfused or fed, as the case might be, there was invariably an increase in the amount of ketone bodies formed by the preparation. If, on the other hand, acids with an odd number of carbon atoms were administered, there was no appreciable increase in ketone-body formation. These results are in excellent agreement with the notion that acetoacetic acid arises by the β -oxidation of butyric acid, which must necessarily be formed by repetitive β -oxidation of the natural fatty acids. In the case of the odd-numbered acids there would be no 4-carbon stage, and propionic acid would be formed instead. Propionic acid is known to be a glucose-former and, as has been pointed out, ketone body formation is always less marked when carbohydrate material is being metabolized.

The general validity of the hypothesis of β -oxidation has been confirmed by experiments in which the phenyl 'markers' used by Knoop were replaced by cyclohexane rings. When the resulting compounds were administered to dogs it was found that derivatives of the even-numbered acids were completely oxidized, those of odd-numbered acids giving rise to benzoic acid. Still more recently it has been shown that if deuterostearic acid, with 18 carbon atoms, is fed to animals, heavy hydrogen can later be found in the palmitic acid (C_{16}) fraction of the body lipoids. Further evidence has been obtained in other experiments to which we shall refer presently and, taken all in all, the evidence in favour of β -oxidation appears to be overwhelming.

Various objections to and criticisms of the work so far described must now be noticed. One evident criticism of work in which fatty acids are substituted by abnormal 'markers' is that new and abnormal reactions may take place as a result of the modification of the chemical structure of the fatty acids. This seems improbable, however, since the use of unsubstituted fatty acids in liver perfusion experiments, and in feeding experiments on phlorrhizinized and diabetic animals, give results which conform so precisely with expectations based on the hypothesis of β -oxidation. So also do experiments with deuterated fats. It may also be objected that Knoop and Embden worked almost exclusively with fatty acids containing 6-7 carbon atoms at most, and these are so much shorter than the 14-18 carbon chains which predominate among natural fatty acids that they might conceivably be metabolized in a different manner. The possibility therefore remains that other types of oxidation might apply when longer chains are being metabolized. A little evidence has been obtained for the occurrence of α -, γ - and even δ -oxidation, but little of it carries much conviction. In more recent times, however, evidence has been brought forward to show that fatty chains can be oxidized at the ω -position. Such a process could not have been detected in Knoop's experiments because the ω -carbon had been protected against oxidation by the introduction of a phenyl radical.

ω -OXIDATION

Evidence in favour of the ω -oxidation of fatty acids was first brought forward by Verkade who, with his colleagues, prepared the triglycerides of octanoic (C_8), nonanoic (C_9), decanoic (C_{10}), undecanoic (C_{11}), dodecanoic (C_{12}) and tridecanoic (C_{13}) acids. These compounds were fed to dogs and to the investigators themselves, the urines being collected and worked up for dicarboxylic acids. The C_{11} derivative, for example, was found to give rise to dibasic acids with 11, 9 and 7 carbon atoms respectively:

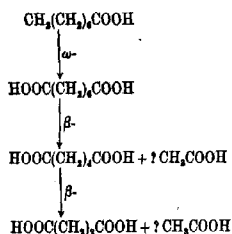


Acids containing more than 12 or less than 8 carbon atoms invoked little or no diaciduria however. Verkade concluded that free fatty acids can be oxidized in the ω -position to yield the corresponding dicarboxylic acids, and that the latter then undergo repetitive β -oxidation from both ends.

That dicarboxylic acids can be formed from fatty acids in the animal body is evident enough from the results of Verkade's experiments. Other workers have administered dibasic acids to dogs and recovered them in part from the urine, together with other dicarboxylic acids with 2 or 4 carbon atoms less. Thus undecandioic acid (C_{11}) gave rise to azelaic (C_9) and pimelic (C_7) acids, while sebacic (C_{10}) yielded suberic (C_8) and adipic (C_6) acids. Dibasic acids with 12–18 carbon atoms were completely oxidized and the same was true of acids with less than 8 carbon atoms. That the acids between these two extremes are excreted presumably means that they are more resistant to oxidation than those with longer or shorter chains.

In view of these results it seems difficult to believe, as some authors do, that ω -oxidation can play no part in the oxidation of fatty acids. It should be remembered, moreover, that in Verkade's experiments and those of his followers, the fatty chains used approximated fairly closely to the naturally occurring chains, among which C_{14} - C_{18} acids predominate. In the work of Knoop, Embden and others, in which ω -oxidation was not detected, the acids employed usually contained from 1 to 7 carbon atoms only, and longer chains were rarely used.

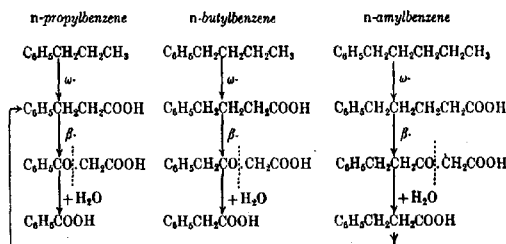
Verkade's conclusions cannot be accepted without reserve, however. With chains of 6 or 8 carbon atoms we should expect that ω - and β -oxidation together would yield one or two 2-carbon fragments together with succinic acid, e.g.



Succinic acid is known to be a glucose-former, while the 2-carbon fragments could perhaps react together and produce one molecule of acetoacetic acid, though in the presence of succinate it is likely that ketone bodies would not appear. Yet there is incontrovertible evidence (p. 399) that octanoic acid can give rise to *two* molecules of acetoacetic acid, and that hexanoic acid, which would be expected to give one 2-carbon fragment and one molecule of succinate, is able to yield $1\frac{1}{2}$ molecules of acetoacetate. Finally, in the case of butyric acid, ω -oxidation would lead directly to the production of one molecule of succinic acid whereas, in fact, the product is acetoacetic acid, often in quantitative yield. There cannot be much doubt therefore that ω -oxidation does not take effect unless the fatty acid chain contains more than 8 carbon atoms or thereabouts, a conclusion that explains why no di-

aciduria was observable when the triglycerides of acids with less than 8 carbon atoms were used in Verkade's original experiments.

How the oxidation of the terminal methyl radical is accomplished we do not know, but that such a group can be oxidized to a carboxyl radical is well established and had, indeed, been demonstrated before Verkade's results were published. *n*-Propylbenzene has been injected intraperitoneally into dogs and found to give rise to benzoic acid in the urine, while *n*-butyl and *n*-amylbenzenes yielded phenylacetic and benzoic acids respectively. These results are completely in accord with the supposition that the side chains of these compounds undergo first ω - and then β -oxidation, exactly as though they were typical fatty acids:



It has been urged against the hypothesis of ω -oxidation that the 4-carbon product arising from combined ω - and β -oxidation of fatty acids of the naturally occurring series would be, not butyric, but succinic acid, so that no explanation would remain for the production of acetoacetic acid and the other ketone bodies to which the latter gives rise. Succinic acid, indeed, is a glycolytic compound and, as such, would diminish rather than encourage the production of the ketone bodies. But this perhaps is not a very serious objection. The majority of the experiments in which massive production of ketone bodies has been demonstrated at the expense of fatty acids involved the use of short-chain acids, usually with 7-8 carbon atoms or less, and to these, as we have seen, it is probable that ω -oxidation does not apply.

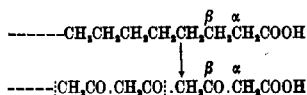
FORMATION OF ACETOACETIC ACID

A great deal of attention has been paid to the problem of ketone-body formation, especially in recent years. Jowett and Quastel, for example, have used the liver-slice method, and with its aid confirmed in a general manner the results of earlier experiments carried out by the older techniques. They found that ketone bodies are formed from fatty acid chains containing an even number of carbon atoms, and that much smaller amounts are produced from odd-numbered chains. In particular they carried out quantitative determinations of the yields of ketone bodies (expressed in terms of acetoacetate) from a series of fatty acids, and discovered that some fatty acids give rise to more than one molecule of acetoacetic acid for each molecule of fatty acid oxidized. Other workers, using a similar technique, obtained rather different figures but, nevertheless, confirmed the observation that, in certain cases, one molecule of fatty acid can produce more than one molecule of acetoacetate. Feeding experiments carried out on starving rats have added further confirmation and, in the case of octanoic acid, which we may take as typical, two molecules of acetoacetate were obtained from each molecule of the C_8 acid. This corresponds to a complete conversion of fatty acid-carbon to ketone-body-carbon.

Now according to the theory of β -oxidation, as formulated by Knoop, a C_8 acid would lose two pairs of carbon atoms in the form of presumptive acetic acid, leaving butyric acid which, undergoing β -oxidation in its turn, would be converted into one molecule *and no more* of acetoacetate. On Verkade's theory of ω -oxidation a C_8 acid would similarly lose two pairs of carbon atoms and yield one molecule of succinate, so that no acetoacetate at all would be expected in this case. Thus neither of these theories could account for the observed behaviour of octanoic acid, and a new explanation had to be sought.

One way of accounting for the observed high yields of ketone bodies is to invoke the theory of multiple alternate oxidation. According to this scheme the fatty chain is supposed to undergo oxidation to form keto-groups at the β -carbon

atom and every alternate carbon atom along the chain, thus:



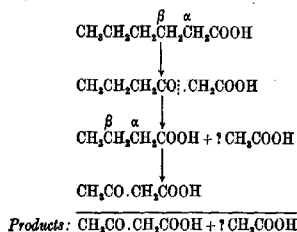
The chain is supposed then to split into 4-carbon fragments, each fragment coming away in the form of a molecule of acetoacetic acid. From every molecule of octanoic acid, therefore, we should expect to obtain two molecules of acetoacetic acid and this, of course, is what is actually observed.

But to accept this hypothesis means totally ignoring and setting aside all the considerable mass of evidence in favour of both β - and ω -oxidation: indeed, the only sound evidence in favour of multiple alternate oxidation seems to consist of precisely those facts which it sets out to explain. Furthermore, there is an alternative possibility which is entirely consistent with the theories of ω - and β -oxidation, i.e. that pairs of the 2-carbon product of β -oxidation react together to form acetoacetate.

It might, of course, be objected that β -oxidation applies as much to odd-numbered as it does to even-numbered chains, so that *all* fatty acids ought to give rise to acetoacetate if the latter is indeed formed from the 2-carbon units, whereas little ketone-body production has ever been observed from odd-numbered chains. In fact, however, most of the experiments on acids of this series were performed only with chains containing 7 carbon atoms or less. These would yield only one or two 2-carbon fragments and one molecule of propionic acid which, being a glucose-former, might be expected to prevent the formation of acetoacetate. In any case it has recently been shown that valeric, heptanoic, nonanoic and undecanoic acids are all ketogenic in rabbits.

If now we consider the case of a fatty acid with less than 8 carbon atoms in the chain we shall expect that only β -oxidation will take place, for there are, as we have seen, sharp indications that ω -oxidation does not assume significant proportions in

chains of this length. In the case of a 6-carbon chain, for example, we should expect the following reactions:



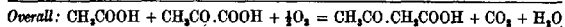
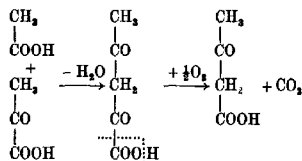
The only possible source of acetoacetic acid here, over and above the single molecule accounted for by Knoop's theory, is the 2-carbon fragment. Even the theory of multiple alternate oxidation cannot account for the production of more than one molecule of acetoacetate in this particular case. Yet it has been shown that all the carbon of hexanoic acid can give rise to acetoacetate.

Experiment shows that butyric acid, with its 4 carbon atoms, yields a maximum of one molecule of acetoacetate. Hexanoic and octanoic acids can yield $1\frac{1}{2}$ and 2 molecules of acetoacetate respectively, but with decanoic and higher acids the amounts of ketone bodies formed are always less than the theoretical maximum. This, presumably, is because ω -oxidation comes into play as longer chains are reached so that, in addition to the usual 2-carbon fragments, succinic acid also is formed and, being a glucose-former, tends to suppress the appearance of ketone bodies.

Clearly it is important to consider the possibility that acetoacetate and the other ketone bodies formed from it may arise primarily from the 2-carbon units removed from the fatty chain by β -oxidation. These, as we have seen, might perhaps consist of acetate. Many experiments have been carried out in order to elucidate the fate of acetate added to various tissue preparations. Feeding, perfusion, tissue-slice and brei experiments have all been used and, while different quantitative data have come out

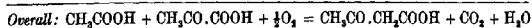
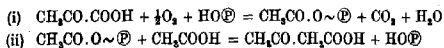
of different experiments, it has been established beyond reasonable doubt that acetic acid is at any rate partially converted into acetoacetate by kidney and other tissues. That part which is not so converted disappears and is presumably oxidized. Moreover, the yields of acetoacetate are greatest in livers that are poor in glycogen and it therefore appears that two possible fates await acetic acid in the liver. Either (*a*) pairs of acetate molecules react together and give rise to acetoacetate, from which the other ketone bodies subsequently arise, or else (*b*) acetate is completely oxidized. The factor determining the extent to which condensation and oxidation respectively shall take place appears to be the availability of carbohydrate. With low-glycogen livers there is much condensation and little oxidation, while in high-glycogen livers there is much oxidation and little condensation. Here, therefore, as in fat metabolism generally, we find that the oxidation of fatty metabolites depends intimately upon the availability of carbohydrate or carbohydrate metabolites.

The mechanism whereby acetoacetate is formed from acetate is still not certainly known. Krebs has produced evidence that acetoacetic acid may be formed from pyruvic and acetic acids together, thus emphasizing the relationship between the metabolism of acetate and that of a carbohydrate metabolite. His observations are consistent with the supposition that acetic and pyruvic acids condense together to form acetopyruvic acid. The latter, being an α -keto-acid, could then undergo oxidative decarboxylation and give rise to acetoacetate:



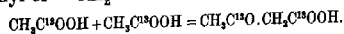
But the results are equally consistent with the supposition that pairs of acetate molecules condense together, forming acetoacetate. It may be that in these experiments Krebs was in

reality obtaining acetic acid in the form of its highly reactive phosphate by oxidative decarboxylation of the added pyruvic acid, and it might be expected that, as acetyl phosphate contains an energy-rich bond, it would condense relatively readily with acetic acid:



The overall results of the two sets of equations are precisely the same, and at present we have not evidence enough to decide between them.

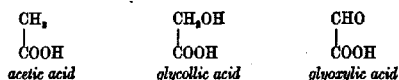
Convincing evidence of the formation of acetoacetate from acetate has recently been obtained by the use of isotopic carbon, for if 'labelled' acetate is incubated with liver tissue and the resulting acetoacetate is isolated, it is found that the isotopic carbon has been transferred to the acetoacetate. The behaviour of octanoic acid in particular has been studied by the isotope method. Octanoic acid was prepared with C^{13} in the carboxyl radical, the product was incubated with rat liver tissue in the form of slices, and the acetoacetate formed was isolated and analysed for C^{13} . Now according to the classical theory of β -oxidation, acetoacetate would be formed only from the butyrate left after two 2-carbon units had been removed, and the product should therefore contain no C^{13} . According to the theory of multiple alternate oxidation, the 8-carbon chain would give rise to two molecules of acetoacetate, of which only one would contain C^{13} , located in the carboxyl group. If, however, pairs of carbon atoms were removed in the form of acetic acid or some highly reactive derivative thereof, C^{13} should be present both in the carboxyl and the ketonic groups of the product, but not in the methyl or $-\text{CH}_2-$ radicals:



It was found that, in fact, isotopic carbon was present in the carboxylic and ketonic, but not in the methyl or $-\text{CH}_2-$ radicals, indicating that the acetoacetate formed must have been produced from the 2-carbon units split off from the molecules of

octanoic acid by β -oxidation. There cannot therefore be much doubt that acetoacetate can indeed be formed from 2-carbon material in the form of acetate or, perhaps, some reactive derivative, possibly acetyl phosphate.

The second possible fate of acetic acid in liver and other tissues is that it may be oxidized, and the oxidative disappearance of acetic acid has recently been studied in experiments in which sliced kidney tissue was used as experimental material. It had previously been suspected that glycollic and glyoxylic acids might be intermediate products in this process on account of their structural relationships to acetic acid:



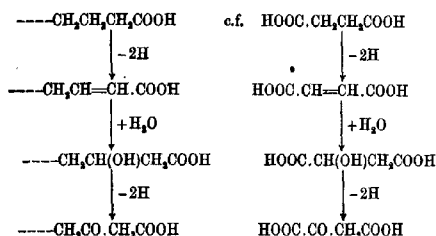
All three substances were tested. Glycollic acid was found not to be appreciably oxidized, while glyoxylic acid strongly inhibited the oxidation of acetic acid. Acetic acid itself was rapidly oxidized, the oxidation being strongly inhibited by malonate. This latter observation suggests that acetic acid itself may undergo oxidation by way of the tricarboxylic acid cycle, a possibility that we have already mentioned (p. 375).

Although the evidence so far presented is insufficient to justify any final conclusions it seems reasonable to frame three hypotheses, viz. (1) that the 2-carbon fragments removed by β -oxidation consist of acetic acid or some highly reactive derivative of acetic acid; (2) that these 2-carbon units are oxidized by way of the tricarboxylic acid cycle; and (3) that when they are not oxidized as fast as they arise, these units condense together to yield acetoacetate, from which the other ketone bodies are then formed. With these hypotheses in mind we may pass on to consider what is known about the intimate details of fat metabolism.

MECHANISMS OF FAT METABOLISM

Very little is known about the intermediate reactions which together make up the process of β -oxidation. There is even some doubt whether β -keto-acids are produced on any significant scale

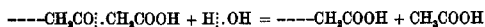
since, apart from acetoacetic acid, no such acids have been discovered in animal tissues. This, admittedly, does not prove that such acids are not formed, for it is always possible that they are transitory products, being removed as fast as they arise. There is a little evidence, as has been seen, that the first stage in the process consists in the introduction of an $\alpha:\beta$ double bond, and by analogy with the reactions involved in the conversion of succinate to oxaloacetate the following reactions might be suggested:



If these reactions correspond to reality it would be expected that the unsaturated, hydroxy- and β -keto-acids would all react similarly in the tissues. These possibilities have been considered by Dakin, who prepared β -ketohexanoic, β -hydroxy-hexanoic, and $\alpha:\beta$ -hexanenic acids, viz. $\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}\cdot\text{CH}_2\text{COOH}$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{COOH}$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}=\text{CH}\cdot\text{COOH}$. When these compounds were perfused through surviving livers, all three behaved similarly and gave similar yields of acetoacetic acid. While these results are in agreement with the sequence of reactions suggested above they do not in any way prove that, even if the unsaturated, the hydroxy- and the β -keto-acids do arise in the course of metabolism, they necessarily arise in that order. Butyric, crotonic and β -hydroxybutyric acids likewise all give rise to acetoacetic acid, but there is considerable doubt whether crotonic acid is formed as an intermediate between butyric on the one hand and β -hydroxybutyric acid on the other.

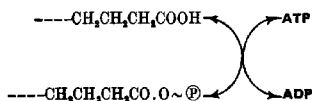
If now we assume that in some way or other β -ketonic acids are indeed formed, we may inquire how they are split into the

2-carbon fragment and the fatty acid with 2 carbon atoms less. Here the classical view was in favour of simple hydrolysis:



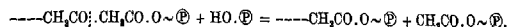
The objections to this scheme are, first, that β -keto-acids are not prone to this kind of hydrolysis at physiological pH, and second, that the biological formation of acetic acid from acetoacetic acid or any other β -ketonic acid in significant quantities has never been demonstrated. Knoop disposed of the latter difficulty by the supposition that acetate is removed as fast as it is formed. It is probable that acetic acid, or some reactive derivative of acetic acid, does arise in the body, since many drugs containing ---NH_2 radicals, e.g. aniline and the sulphonamides, are excreted in acetylated form after being administered to experimental animals. Moreover, as has been pointed out already, small amounts of acetic acid have been isolated from liver tissue in the form of the 2:4-dinitrophenylhydrazide.

Recent discoveries concerned with the breakdown of carbohydrate have shown that the initial stages involve 'priming' of the substrate by the transference to it of energy and phosphate radicals from ATP. Evidence, mostly unconfirmed as yet, is accumulating to show that something similar may take place in the breakdown of fatty acids. Already it has been shown by Lehninger that acylphosphates are more readily attacked by the liver than are the free acids, suggesting that phosphorylation may be an essential preliminary to β -oxidation. Since the carbonyl phosphate radical contains an energy-rich bond, $\text{>C.O} \sim \text{P}$, it is probable that the 'priming' process must be carried out by group transfer from ATP:



On account of its larger free energy, the resulting acyl phosphate might well be more susceptible than the free acid to dehydrogenation and the other stages involved in β -oxidation.

Assuming that the phosphorylated fatty acids undergo β -oxidation to yield the corresponding β -ketonic acids, there arises the question of how the 2-carbon fragment is split off. At the time of writing there is evidence, amounting to little more than a suspicion, that the splitting is phosphorolytic rather than hydrolytic:



This is an interesting possibility. It means, in effect, that the 'priming energy' would be recovered in the energy-rich bond of acetyl phosphate, while the residual fatty chain would be left already 'primed' for the next step in the degradation. Further, the production of acetyl phosphate in place of acetic acid itself means that a highly reactive product is formed in place of a comparatively inert 2-carbon fragment, and the condensation of pairs of acetyl phosphate molecules to yield acetoacetic acid might be expected to take place readily. Again, there is evidence that when acetate is oxidized it does so through the tricarboxylic acid cycle, into which it may enter in the first instance in the phosphorylated form. In any case, the newer ideas make it seem less surprising than hitherto that attempts to isolate the 2-carbon product of β -oxidation have met with little success.

Tentatively, then, we may suppose that the first stage in the oxidation of fatty acids consists in their phosphorylation, most likely at the expense of ATP. Subsequent β -oxidation and phosphorolysis yields 2-carbon fragments in the form of acetyl phosphate. The acetyl phosphate units arising in β -oxidation can either (a) undergo oxidation or (b) condense together to give acetoacetic acid. How ω -oxidation takes place we are still unable to say.

We have already mentioned some facts which point to the tricarboxylic acid cycle as the mechanism through which acetate undergoes biological oxidation, viz. (a) that when pyruvate is oxidized through this cycle the probabilities are strong that it undergoes preliminary oxidative decarboxylation to give acetate (p. 375) and (b) that the oxidation of acetate itself is inhibited

by malonate (p. 404). It may now be added that, according to Breusch, the incubation of β -keto-acids with oxaloacetate in the presence of an enzyme, 'citrogenase', which can be obtained from kidney, brain or muscle, leads to a large-scale synthesis of citrate, together with a fatty acid containing 2 carbon atoms less than the parent keto-acid. There are clear indications here that the terminal pair of carbon atoms leaves the keto-acid and unites with oxaloacetate, presumably to yield aconitate in the first instance. Similar results have been reported from Wieland's laboratory and these can be interpreted on the same lines, though the workers themselves have suggested other interpretations. We know that citrate itself does not lie on the tricarboxylic acid cycle and that its accumulation is an artefact brought about by experimental conditions that block later reactions in the cycle. Perhaps we are justified in concluding, in view of all the evidence, that the terminal pair of carbon atoms is removed, possibly by phosphorylase, and enters into combination with oxaloacetate to yield aconitate in the first instance and that the rest of the reactions of the tricarboxylic acid cycle follow, oxaloacetate being regenerated while the two carbon atoms appear in the form of carbon dioxide. These reactions are already known to yield a rich harvest of energy-rich bonds.

The entry of the 2-carbon substance, acetate or acetyl phosphate, into the cycle is conditional in the liver, though not perhaps in other tissues, upon the availability of carbohydrate, since oxaloacetate is required to gain access to the cycle. In the liver, where β -carboxylase is present, oxaloacetate is in equilibrium or near-equilibrium with pyruvate and carbon dioxide. Pyruvate in its turn is in equilibrium with glycogen through a long series of intervening equilibria, and it follows that, when carbohydrate is in short supply, the concentration of pyruvate will tend to be low and that of oxaloacetate to be low also as a result. Under these conditions the 2-carbon units will be able to enter the cycle and be metabolized only slowly. Now when carbohydrate metabolism is subnormal there is usually a correspondingly heavy emphasis upon that of fat, leading to a proportionately greater formation of acetate (or acetyl phos-

phate). It follows, therefore, that in the absence or deficiency of available carbohydrate there will not only be a tendency for the 2-carbon units to accumulate for lack of oxaloacetate, but a simultaneous increase in their rate of production as well. But we know now that acetate (or acetyl phosphate) can give rise to acetoacetate, and this may be expected to happen under conditions in which acetate (or acetyl phosphate) tends to accumulate, i.e. under conditions which are characterized by subnormality of carbohydrate metabolism. At last, therefore, we can offer a reasonable explanation of the long-familiar fact that ketone bodies tend to be formed when carbohydrate and carbohydrate metabolites are in short supply, a relationship that hitherto has been inexplicable.

METABOLISM OF ACETOACETIC AND β -HYDROXYBUTYRIC ACIDS

Much attention has been given to the problem of the metabolism of acetoacetic and β -hydroxybutyric acids, and it is now well established that they are interconvertible through the action of β -hydroxybutyric dehydrogenase together with Co I, both of which are widely distributed in animal tissues. Remarkably little more is known.

Many tissues are able to oxidize added acetoacetate and β -hydroxybutyrate, but practically nothing is certain about the manner in which these oxidations are accomplished. Among the most recent contributions to this subject is the work of Kleinzeller, who studied the behaviour of a group of 4-carbon acids related to the ketone bodies in the presence of sliced kidney tissue, a material that was selected because of its known ability to oxidize acetoacetate to carbon dioxide and water. The compounds examined included the following:

Butyric acid	$\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}$
Crotonic acid	$\text{CH}_3\text{CH}=\text{CH}\cdot\text{COOH}$
β -Hydroxybutyric acid	$\text{CH}_3\text{CH}(\text{OH})\text{CH}_2\text{COOH}$
γ -Hydroxybutyric acid	$\text{CH}_3(\text{OH})\text{CH}_2\text{CH}_2\text{COOH}$
α - γ -Dihydroxybutyric acid	$\text{CH}_3(\text{OH})\text{CH}_2\text{CH}(\text{OH})\text{COOH}$
Vinylacetic acid	$\text{CH}_2=\text{CH}\cdot\text{CH}_2\text{COOH}$

substances which, like butyrate, crotonate and β -hydroxybutyrate, are known to give rise to acetoacetate, can also be accounted for, while the mechanism postulated here would at the same time explain Kleinzeller's observation that the oxidation of these substances is malonate-sensitive.

It remains to be explained why acetoacetate, β -hydroxybutyrate and related compounds are freely oxidizable in certain tissues other than liver, even when carbohydrate is in short supply. Unlike the liver, kidney and muscle contain no β -carboxylase, so that oxaloacetate is only spontaneously (as opposed to catalytically) degraded to pyruvate and carbon dioxide, and its concentration in these tissues is not immediately dependent upon the availability of carbohydrate. How these tissues maintain their stocks of oxaloacetate is not known, but it has been found that the addition of insulin maintains the respiration of pigeon-breast muscle, suggesting that this hormone must in some way be connected with the production or preservation of oxaloacetate in the extrahepatic tissues. A further point that is probably significant is Breusch's observation that the 'citrogenase' activity of kidney, brain and muscle is much higher than that of liver, suggesting that 'citrogenase' includes not only some enzyme that catalyses the condensation of acetate or acetylphosphate with oxaloacetate, but some factor which at the same time produces or preserves oxaloacetate itself.

It must be emphasized that our ideas here are as yet hardly out of the speculative phase. We have evidence enough only to be suggestive. In the last few pages we have taken what evidence is available at the time of writing and used it in a frankly speculative manner, trying, nevertheless, only to frame hypotheses that are reasonably consistent with the evidence and with general principles, and thus to gain a coherent idea of the ways in which the intimate business of fatty acid metabolism is perhaps conducted. The field is being studied intensively at the present time and important advances may confidently be expected.

Evidence is accumulating to show that the metabolism of fatty acids, like that of carbohydrates, involves phosphate radicals at many points, and that the oxidative degradation of

This hypothesis, at best, has always been purely speculative and with no factual basis, apart from the fact that aldehydes readily undergo aldol condensation under *in vitro* conditions. There is no reason to believe that acetaldehyde arises in quantity in the tissues of animals. Despite a complete lack of evidence it has often been suggested that acetaldehyde might arise, as it does in yeast, by the action of carboxylase upon pyruvic acid; this latter suggestion, indeed, has been thought to provide an explanation for the synthesis of fats from carbohydrate sources.

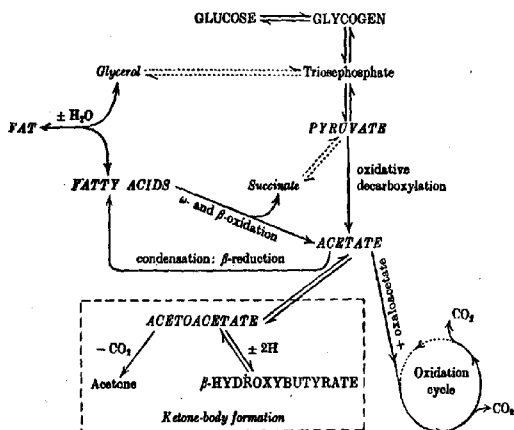


Fig. 34. Outlines of fat metabolism. No intermediate stages are shown; many of these probably involve phosphorylated intermediates.

Recent developments in our ideas about fat metabolism suggest an alternative hypothesis which, while it is at present hardly less speculative than the aldol mechanism, has at least the virtue of being reasonable. It may be suggested that the starting material is, not acetaldehyde, but acetyl phosphate. The latter is known to be derivable from pyruvic acid by oxidative decarboxylation and, in addition, is probably a primary product of the β -oxidative fission of fatty acid chains. Pairs of acetyl

glucose when administered to diabetic or phlorrhizinized animals. Apart from glycerol and succinate, however, fats are not known to give rise to anything but acetic acid and the ketone bodies which appear to be derived from it.

Experiments have been performed in which the incubation of sliced rat liver with butyric acid gave small but apparently significant increases in the glycogen content of the tissue. But, as we now know, glycogen and fat alike are oxidized through a common metabolite, acetic acid, and it may well be that the addition of butyrate merely spares the oxidation of glycogen by giving rise to this common metabolite.

Since acetate can be formed by the oxidative decarboxylation of pyruvate and can itself give rise to the synthesis of fat, there is not much doubt that this is an important route for the production of fat from carbohydrate sources. It may be asked whether this process is reversible. There is, however, no evidence that the stage of oxidative decarboxylation can be reversed, so that while pyruvate may give rise freely to acetate, acetate is not convertible into pyruvate. Buchanan has shown that, if lactate or pyruvate containing isotopic carbon is administered to animals, the carbon isotope can be partly recovered in the liver glycogen. If acetate similarly 'labelled' is used instead, however, practically no transference of the isotope to the liver glycogen can be detected. It may therefore reasonably be assumed that the conversion of acetate to pyruvate takes place to a limited extent at most if, indeed, it takes place at all.

In short, there is no reliable evidence for any large-scale production of carbohydrate from fatty sources and, in so far as such a conversion takes place at all, it must probably be attributed to the formation of glycerol and succinate as intermediary metabolites of fat katabolism.

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The list of references that follows makes no claim to be in any sense complete or exhaustive. It is designed only as an introduction to the literature, and a wealth of further references will be found in the books (marked with an asterisk) and review articles quoted. Preference has been given on the whole to recent publications, but for the most recent work the *Annual Reviews of Biochemistry* and the *Annual Reports of the Chemical Society* may profitably be consulted. Here will be found many valuable and critical appraisals of the newest work, together with exhaustive lists of references.

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